EARLY DEVELOPMENT IN THE CELLULAR SLIME MOULD

DICTYOSTELIUM DISCOIDEUM

by

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Except when stated otherwise the work presented in this thesis is my own. The major part of it has been published in collaboration with Dr. Marilyn Monk (J. gen. Microbiol., 1974, 85, 321-334) and with Dr. George Bazill (J. gen. Microbiol., 1975; in press).

This work is dedicated to my family and to my friends, to all those who through love, understanding and encouragement made it possible and enjoyable.

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Felix qui potuit rerum cognoscere causas ....

Virgil. Georg.II 490

ABSTRACT

This thesis is concerned with the pre-aggregation and aggregation stages of the developmental cycle of the cellular slime mould Dictyostelium discoideum.

The concentric wave pattern of aggregation was studied in an attempt to clarify the mechanism of intercellular communication. The analysis of this pattern by direct microscopic observation and in time-lapse films led to the following conclusions:

1. The relayed signal has a range of approximately 57 µm; this width of the relay zone includes one or more cell rows depending on the population density.
2. The signal relay time (directly measured) is approximately 12 sec.
3. The velocity of signal propagation varies from 4.70 to 6.89 µm/sec (mean velocities) when the population density varies from $5 \times 10^5 - 5 \times 10^4$ cells/ cm 2. The variation of velocity is thus a slow function of population density.
4. The concentric wave pattern results from the succession of alternate bands of moving and still cells corresponding to the propagation of signals emanated from the aggregation center. The width of the movement bands reflect the distance the signal is propagated during the period of movement response (100 sec) of a single cell; the width of the still bands (interbands) depend upon the distance is propagated between signal the signal/events and varies with signal frequency. The agreement between the values of signal velocity calculated on the basis of six distinct parameters of the concentric wave pattern supports this interpretation.
5. The aggregation signal is pulsatile and its frequency

increases during aggregation.

6. The refractory period for the movement response is not greater than 12 sec. This finding is discussed in the light of both the sustained (100 sec) directionality of the aggregation movement steps and the relay time of 12 sec.

The duration of the pre-aggregation stage is population-density-dependent. This observation prompted a series of experiments of which these are the pertinent conclusions:

1. During pre-aggregation the myxamoebae secrete into the medium a substance (acceleration factor, AF) which when added to freshly harvested cells at the beginning of development reduces the duration of the pre-aggregation stage to 45 - 65% of its duration in controls.
2. On ion-exchange chromatography, gel filtration and disc electrophoresis AF co-fractionates with the low Km extracellular cyclic-AMP-phosphodiesterase.
3. AF activity depends exclusively on the extracellular activity of the enzyme as a phosphodiesterase and not on a second active site in the molecule.
4. The stimulatory effect of the presence of the cyclic-AMP-phosphodiesterase is related to the removal of cyclic-AMP from the medium and not to the accumulation of its products of digestion.
5. The decrease in the duration of the pre-aggregation phase is due to acceleration of the rate of differentiation toward the aggregation-competence state.
6. The added cyclic-AMP-phosphodiesterase is particularly active as AF during the first half of pre-aggregation.
7. The early period of differentiation toward aggregation-competence is reversible.

These results are discussed in the light of recent work in other laboratories

List of abbreviations:

cAMP	3',5'-adenosine monophosphate.
cGMP	3',5'-guanosine monophosphate
5'AMP	5'-adenosine monophosphate
PDE	3',5'-adenosine-monophosphate-phosphodiesterase
mPDE	cell-membrane-bound form of PDE
ePDE	extracellular form of PDE
$t_{0,1,...n}$	time elapsed since initiation of development (hours)
t_{agg}	time of onset of aggregation
AF	acceleration factor of aggregation
MB	width of a movement band
IB	width of an interband

TABLE OF CONTENTS

CHAPTER 1

Introduction

1. The life cycle of Dictyostelium discoideum

Dictyostelium discoideum is a member of the group Acrasiales. These are cellular slime moulds (Shaffer, 1953) which means that although they pass through social phases their life cycles do not involve cell fusion and formation of true plasmodia. They are eukaryotic organisms without a formal sexual cycle (Clark et al, 1973; Erdos et al, 1973).

D.discoideum is a haploid organism (Brody & Williams, 1974) which lives naturally in damp soils or on rotting leaves, feeding on bacteria. It was first described by Raper (1935) who subsequently published a detailed report on its life cycle supplemented with data of experiments on excision and grafting in the pseudoplasmodium (Raper, 1940). In the laboratory it is grown on solid media or in suspension, in both cases associated with bacteria (Aerobacter aerogenes and E.coli B/r are the more commonly used) which are phagocytosed into food-vacuoles (Gezelius, 1959). The generation time in these conditions is about 3 hours and the plating efficiency from isolated cells is approximately 100 per cent (Newell, 1971). During growth each cell divides by fission and the resulting individuals (10-15 μm in diameter) move apart (amoeboid movement) showing, on random collision, the characteristic behaviour of contact-inhibition of movement: pseudopods are retracted at the point of contact and the direction of movement is changed. The food-seeking process involves chemotactic mechanisms. Two attractants have been identified in bacterial extracts: cAMP (Konijn et al, 1967) and folic acid (Bonner et al, 1970; Pan et al, 1972); the latter, although having a smaller rate of diffusion than cAMP, is more efficient in the attraction of vegetative cells (Bonner et al, 1970). Increased sensitivity to cAMP is acquired only during the early phases of the development cycle (Bonner et al, 1969).

In order to facilitate biochemical studies several workers have isolated axenic strains (Sussman & Sussman, 1967; Schwalb & Roth, 1970; Watts & Ashworth, 1970; Loomis, 1971); these strains grow, in agitated cultures, on peptone and yeast extract media which may be supplemented with sugars. Axenic growth is a recessive character coded by two genes located in two of the seven linkage groups (Brody & Williams, 1974; Williams et al, 1974 a; 1974 b). During axenic growth the generation time (Ax_2 strain) is about 12 hours decreasing in different cultures to about 8 hours (personal observation). Axenic strains grow more slowly than non-axenic strains even when cultured with bacteria (Williams et al, 1974 a; 1974 b). No defined medium has yet been developed.

The developmental cycle is triggered by lack of food (Arndt, 1937) and the amoebae change then from individualistic to social behaviour. Ax_2 and NC_4 strains grown in shaken suspensions (respectively axenically and on bacteria) are inhibited from switching to the social phase by substance(s) which are present or accumulate in the medium; washing and resuspension in non-nutrient solutions is required to start differentiation. Development starts with a period of differentiation during which the cells acquire competence for aggregation (see section 4); this period is designated "pre-aggregation" or "interphase" (Bonner, 1963). The duration of this period varies according to the density of the population (see "Results" in this thesis); an average value for NC_4 and Ax_2 strains is about 8 hours. During pre-aggregation the cells move randomly, actively at first, then progressively slowing down and adhering to each other (personal observation).

The aggregation phase starts when the myxamoebae move coordinately in response to signals emanating from localised points which subsequently become central points of collection; there they form, after a period of about 5 hours, a multicellular body which may contain from 12 to 10^5

cells (Gerisch, 1968). Each aggregate will collect cells from an area with an average radius of 1.27 mm (Bonner & Dodd, 1962). During the centripetal movement the myxamoebae establish the specific contacts which are essential for morphogenesis. The phase of aggregation is described in more detail in section 2.

Once an aggregate is formed further development depends upon the presence of an air-water interface (Gerisch, 1968). A tip, a pointed protuberance, develops locally at the interface and functions as an organiser in all subsequent stages of development (Bonner, 1950; Raper, 1940; Robertson et al, 1972 b; Farnsworth, 1973). In Farnsworth's elegant study on tip induction and dominance it was shown that a tip inhibits formation of second tips possibly through diffusion of one or more substances (the insertion of an impermeable barrier in an aggregate for at least 30 minutes overcame the inhibition); the generation of another tip when the original one was removed took an average of 32 minutes. Furthermore, this author observed that only at a particular stage is the aggregate competent to form a tip.

The tipped cell mass rises in a slender, conical shape, the "conus", which develops according to an axis whose orientation is related to the position of the tip (Robertson et al, 1972 b; Farnsworth, 1973). The conus may either evolve directly to a fruiting body or fall down on the substratum and migrate ("slug" stage). The pattern of co-ordinated movement which causes erection of the conus and migration is unknown but it has been shown by grafting experiments that migration does not involve generalised cell redistribution (Raper, 1940). By grafting fluorescently marked tips on unlabelled cell masses from which the original tip had been removed, Farnsworth (1973) demonstrated that the majority (70%) of tip cells stay at the tip during the period of

migration. There are suggestions, based on the absence of chemotactic ability towards cAMP in slug cells (Garrod & Malkinson, 1973a; Garrod, 1974) that migration is not controlled by the mechanism of aggregation. However it was reported, in contradiction to that suggestion, that both migration and the ultimate stage of erection of the fruiting body (culmination) like the aggregation movement, are periodic movements (Robertson, 1972; Robertson et al, 1972 b); moreover it appears that the tip secretes the aggregation attractant throughout development as shown by the response of sensitive pre-aggregation cells to transplanted tips (Robertson et al, 1972 b; Rubin & Robertson, 1975). It was found (Bonner, 1949; Garrod & Malkinson, 1973a) that the anterior one tenth of the slug produces most of the cAMP (> 50%) synthesised by the slug; if the mechanism of chemotaxis functioning during aggregation applied to the stage of migration one would expect cAMP concentration to show no decrement along the slug axis. The nature of the control of cell movement at the late stages of development is therefore still obscure. Migration is not essential in the process of differentiation but, being controlled by elements of the environment such as temperature, humidity, ionic strength and light, it offers obvious advantages on ecological grounds.

The final stage in the process of development, which takes about 7 hours (total duration of development in laboratory conditions is about 24 hours), is that of erection of a small (about 1 mm in height) fruiting body. This is formed by a spherical mass of ellipsoid spores held aloft by a thin column of vacuolated and turgid cells encased in a cellulose sheath. Culmination involves secretion of an initially internal cellulose sheath formed during the upward movement of pre-stalk cells (Bonner, 1963); this movement is reversed immediately below the tip

and the pre-stalk cells enter the growing column which constitutes the stalk. It has been demonstrated that cellulose tubes can make any cell of a cell mass enclosed in them differentiate into a stalk cell (Farnsworth, 1973); this author suggested that this is not due to direct induction by cellulose (sheets or rods of cellulose have no effect on the pattern of differentiation) and that the induction is related to the selective filtering of certain molecular species. Farnsworth also suggested that the control of the fixed proportion (1:2) of stalk cells to spores regardless of the size of the pseudoplasmodium (Bonner & Slifkin, 1949; Bonner, 1957; Bonner & Dodd, 1962) results from the very process of culmination. However, although attractive, this hypothesis requires knowledge of two essential control mechanisms of which we are equally ignorant; the control of secretion of the cellulose sheath and the control of the mechanism and extent of the ascending movement. In other words, what, according to the proposed mechanism, would stop all the slug cells from becoming stalk cells? That this cannot be the only control of late differentiation is made clear by the ultrastructural evidence that the slug already contains two cell types at a stage of incipient differentiation: pre-stalk cells at the anterior end and pre-spores forming the two rear thirds (Takeuchi, 1963; Hohl & Hamamoto, 1969; Maeda & Takeuchi, 1969).

Evidence has been accumulated in favour of a process of sorting-out amongst cells of a heterogeneous population which would function at the immediate post-aggregation phase (Bonner, 1957; Takeuchi, 1963; 1969). Such a process would segregate the two cell types according to transient characteristics resulting from previous conditions of growth or from conditions of the early development. However

the capacity for repeated regulation following excision of increasing proportions of slug cells and the capacity to form fruiting bodies with as little as 12 cells makes it unlikely that this mechanism plays an essential role in the process of differentiation. The spores, the final viable product of the developmental cycle, are heat and drought resistant. In favourable conditions of humidity and temperature the spores germinate and release actively moving amoebae, ready to feed and grow; in the absence of food a new developmental cycle is initiated (Bonner, 1967).

During development only residual cell divisions occur (mainly during the initial stages) but without increase in mass (Shaffer, 1962). This constitutes one of the great advantages of studying development in D.discoideum: the phenomena of growth and differentiation are naturally separated in time. In the absence of growth, the energy involved in the process of differentiation is obtained exclusively from the breakdown of cell components which takesplace, to a large extent, in autophagic vacuoles (Maeda & Takeuchi, 1969).

In the absence of a sexual cycle the genetical analysis of this organism has been based on the parasexual system of diploidisation and haploidisation associated with mitotic crossing-over (Sinha & Ashworth, 1969; Katz & Sussman, 1972; Williams et al, 1974 a; 1974 b). Diploids may arise spontaneously in laboratory conditions (Ross, 1960; Sussman & Sussman, 1963) or can be specially selected (Loomis & Ashworth, 1968; Loomis, 1969; Sinha & Ashworth, 1969; Katz & Sussman, 1972; Williams et al, 1974 b).

For a critical review on slime moulds see Bonner (1967).

2. Some aspects of the process of aggregation

2-1. The attractant. The first evidence that the process of aggregation in Dictyostelium depends on the diffusion of a chemical secreted into the medium was reported by Runyon (1942): a cell population reproduced the pattern of aggregation of another population from which it was separated by a dialysis membrane. This was confirmed by the experiments of Bonner (1947), conducted under water, showing that attraction could occur around corners and across gaps artificially introduced in the substratum. He called the unidentified attractive substance "acrasin", a name that persisted for more than two decades until the chemical nature of the attractant was discovered. Shaffer (1953) isolated the acrasin from the intercellular medium and showed its capacity to attract sensitive cells experimentally. He also reported (Shaffer, 1956 a, confirmed by Sussman et al, 1956), that acrasin was rapidly inactivated presumably by enzymatic action ("acrasinase").

The discovery of cAMP as the chemotactic agent bringing about aggregation in Dictyostelium discoideum followed an indirect and unpredictable course. First it was identified as an attractant present in bacterial extracts after the observation that bacterial colonies were able to divert aggregation-competent cells (Konijn et al, 1967). cAMP had been shown to function as a mediator of hormonal action both in Vertebrates and Invertebrates (Sutherland & Rall, 1960) and its role in the control of enzymatic activity had been recently extended to bacteria: it was reported that in presence of glucose, cultures of E.coli excrete cAMP into the medium, the intracellular cAMP level decreasing simultaneously (Makman & Sutherland, 1965). This prompted the suggestion by Barkley (Konijn et al, 1968) which led to the identification of the bacterial attractant. Acrasinase activity frustrated efforts to isolate cAMP from the suspension medium of Dictyostelium

discoideum with the result that Polysphondylium pallidum was the first slime mould detected as a natural producer of cAMP (Konijn et al, 1968); ironically this species is not attracted by cAMP but D.discoideum responds to both P.pallidum aggregates (Shaffer, 1953; 1957 b) and to the isolated cAMP. Barkley (1969) was able to separate and identify cAMP in the extracellular medium of D.discoideum, preventing its hydrolysis by PDE (Chang, 1968) by dialysis or by absorption of cAMP to Dowex 1 Cl^-. It was thus proved that cAMP is a strong attractant of D.discoideum and that these cells are able to secrete it; moreover, the rate of secretion is increased during aggregation (Bonner et al, 1969; Malkinson & Ashworth, 1973 a; Klein & Brachet, 1975).

The specificity of the attractant was tested by Konijn et al (1969) and Konijn, (1972) who showed, in disagreement with Chassy et al (1969 a), that all other nucleotides and cAMP analogues are less active than cAMP in stimulating chemotaxis. This has been confirmed by nucleotide-binding assays (Malchow & Gerisch, 1973 b; 1974). Konijn (1972) demonstrated that changes in the base, sugar or phosphate moities of cAMP all reduce the chemotactic activity although in different degrees.

The sensitivity to cAMP increases by a large factor ($\sim 10^2$) during interphase (Bonner et al, 1969). This is paralleled by the increment in the number of cAMP-receptors at the cell surface (Malchow & Gerisch, 1974). Aggregation-competent cells respond to cAMP concentrations ranging from 10^{-9}M to 10^{-4}M (Konijn, 1972). The short-lived, transient binding of cAMP (less than 5 sec) suggested its rapid turn-over at the cell surface (Malchow & Gerisch, 1973 b). It has been shown that, in fact, it is degraded into 5' AMP by ePDE and mPDE; a cell-bound phosphatase, already present during growth, converts a small part (5-25%) of 5' AMP into adenosine. Both are released to the medium

(Malchow & Gerisch, 1974) contrary to the original hypothesis that cAMP might be re-cycled (Cohen & Robertson, 1972).

Malkinson & Ashworth (1973a) followed the kinetics of synthesis and secretion of cAMP during the life cycle of D.discoideum. Secretion is initiated during growth, its concentration in the extracellular medium being proportional to the density of the population. During the stationary phase of growth in axenic medium (Ax_2 strain) the concentration of extracellular cAMP reaches 10^{-6}M, the intracellular cAMP doubling to a concentration of 4×10^{-6}M; this increase in intracellular-cAMP content when growth is arrested, is common to other cell types. The cellular cAMP content is further and progressively increased during pre-aggregation and early aggregation (see also Bonner et al, 1969) reaching a maximum ($\sim 10^{-4}$M) before completion of aggregation; it declines then to values comparable to those of early pre-aggregation. The variation in the concentration of extracellular cAMP during the same period of time follows, with a lag of about one hour, the variation in intracellular cAMP, the peak value being about 7×10^{-7}M at late aggregation. These are however approximate average values since it has been shown (Gerisch et al, 1974 a, b; Roos et al, 1975) that cAMP is synthesized and released periodically into the medium. Nevertheless the specific activity of adenyl cyclase, which converts ATP into cAMP, is constant (3.4 nmoles cAMP/mg protein/min) in vitro, throughout growth and development (Rossomando & Sussman, 1972); this indicates that the observed increase in cAMP production in the first stages of development involves a process of activation of adenyl cyclase. Synthesis and destruction (see section 3) of cAMP is therefore controlled during development. Malkinson & Ashworth (1973a), in an attempt to explain a second peak of extracellular cAMP occurring

about the stage of culmination which is not accompanied by a parallel increase in intracellular cAMP, suggested that the secretion of cAMP might be independently controlled. Klein & Brachet (1975) showed that progesterone stimulates the rate of secretion of cAMP (and PDE) while the intracellular level is kept low; EDTA has an even more marked effect: there is a precocious and intense secretion of cAMP, and the intracellular cAMP concentration increases 4-fold in comparison with controls at the time of aggregation competence. The significance of these findings in relation to possible mechanisms of cAMP secretion is not clear.

It is not known how cAMP is accumulated in the extracellular medium from the first stages of pre-aggregation. Given the importance of this period in the course of development of aggregation-competence (Gerisch et al, 1975 a; Darmon et al, 1975; this thesis) it is of interest to know whether cAMP is already secreted in pulses at this early stage (as in late pre-aggregation; Gerisch & Hess, 1974 b) and whether the accumulation of cAMP is due to an increased rate of pulsation and/or to the increased amplitude of the pulses.

It has been shown that pulse-like stimulation by cAMP accelerates early differentiation (Gerisch et al, 1975a; Darmon et al, 1975); cAMP is also the attractant and the signal transmitter in aggregation (Robertson et al, 1972a; Gerisch & Hess, 1974 b; Roos et al, 1975; Shaffer, 1975). It appears therefore that the whole of early development (comprising the pre-aggregation and aggregation stages) is controlled by cAMP signals.

Reports on a second chemotactic agent still await clarification. Sussman (1958) showed that on fractionation of acrasin by paper chromatography one of the fractions potentiated the chemotactic response to a second one; it was suggested (Rossomando & Sussman, 1973) that

these two fractions might be cAMP and 5'AMP. Bonner et al (1966) reported the occurrence of a "rate substance" with particular high activity in the first five minutes of interphase. The extracellular medium was collected free of cells and after immediate boiling it was assayed for stimulation of motion in cells of Dictyostelium. The rate of movement induced by the early samples increased from 0.35 μm/h (control) to 0.79 μm/h. It has been reported (Konijn et al, 1968) that cAMP increases the rate of movement and, as the "rate substance", it also induces cell adhesiveness. In spite of the conflicting quantitative data (compared with the values of extracellular cAMP concentration published by Malkinson & Ashworth, 1973a and Bonner et al, 1969) it appears that the rate substance, as suggested by Bonner and collaborators themselves, is cAMP (acrasin). Gerisch (1968) described two natural chemotactic agents the discrimination of which was only possible by the behaviour of two mutants. One of the agents is reported to decrease the rate of movement by stimulating production of pseudopods in a more or less uncontrolled way even in wild-type cells; it was designated CF I (chemotactic factor I) and is produced periodically by centers of aggr 50. The second factor (CF II) increases the rate of movement and is produced continuously by the ap 66 mutant. In the absence of further evidence for the two factors I suggest that aggr 50 also emits cAMP but the signal is defective either in its amplitude or periodicity, in a way that disturbs the response even in wild-type cells; it would be hard to conceive the utility of such a signal during normal aggregation. CF II is probably again cAMP emitted by a continuous or high frequency source.

In spite of the almost universal secretion of cAMP among the Acrasiales, cAMP functions as an attractant only for some species of

Dictyostelium (Cohen & Robertson, 1972; Gerisch et al, 1972). The existence of different acrasins became apparent since the early observations (Raper & Tom, 1941; Shafer, 1953; 1957 a) that D.discoideum and P.violaceum, when mixed, segregate themselves out and move towards their own centers of aggregation. Among the cAMP-sensitive species (D.discoideum, D.purpureum, D.mucuroides and D.rosarium) segregation occurs only after joint aggregation; the signal appears to be periodic in all of them, but of different frequencies (Olive, 1902; Raper & Tom, 1941; Cohen & Robertson, 1972; Konijn, 1972). It was reported (Cohen & Robertson, 1972) that the signal is also periodic in P.violaceum (P=80 sec) and it was suggested that the signal may be thus related to a metabolite involved in the system of the cAMP oscillator (Goldbeter, 1975). In the species of Dictyostelium not sensitive to cAMP (D.minutum, D.vinaceumfuscum, D.aureus) the attractant is also a small molecule which diffuses rapidly (Konijn, 1972).

The significance of the secretion of cAMP by species for which it is not an attractant is still unknown. It would be of interest to test if cAMP pulses stimulate development in these cases.

2-2. Centers of aggregation. It is still unclear whether the centers of aggregation in Dictyostelium discoideum are originally single cells. In D.discoideum, unlike both species of Polysphondylium and D.minutum, no special cells (founder cells) could be detected either on a morphological or behavioural basis (Gerisch, 1968; Cohen & Robertson, 1972). This was subject of a long controversy since Sussman & Noel (1952) postulated that populations of D.discoideum contained two distinct types of cells: the initiators and the responders. This hypothesis was based on the observation that the size of the territories of

aggregation (and thus the number of centers) was density-dependent, the number of aggregates being maximal at a density of 2×10^4 cells/cm2. Subsequently the initiators were described as large and specially active cells present in a ratio of about 1/2000 among the responders (Ennis & Sussman, 1958; Sussman & Ennis, 1959). This was contradicted by the work of Bonner and collaborators (Bonner & Dodd, 1962; Bonner & Hoffman, 1963) who found no evidence for density-dependance of aggregation territory size and specially by the work of Konijn & Raper (1961). These authors compared the theoretical probability of aggregation in small populations predicted by the 1/2000 ratio with experimental data and showed that this stage of development is not dependent on special cells, present in a fixed proportion in the population. This conclusion is confirmed by the work of Gerisch (1961).

Shaffer (1956 a, b; 1962) suggested that centers of aggregation corresponded to the first cells to secrete the attractant with a supraliminal amplitude. Aggregation centers may thus be originally single cells that either matured first (Konijn & Raper, 1961; Shaffer, 1962; Bonner et al, 1969; Robertson, 1972) or that are better situated within the cell community (Konijn & Raper, 1961). The equilibrium instability theory expounded by Keller & Segel (1970 a,b) predicts that no initiator cells are necessary (although they may exist) to start aggregation; conditions for the initiation of aggregation could be created by the interplay of three variables: chemotactic sensitivity, non-chemotactic random motion and rate of production of acrasin (in fact they should include a fourth variable, the rate of decay of acrasin, here considered a constant). Instability initiated by either external influences, imperfect synchrony or by the presence of initiator-cells may induce a positive feed-back leading to aggregation. Gerisch (1961;

1968) observed that aggregation centers are formed usually by a curling movement of the first short streams; in view of the recent findings on periodic and synchronous chemotactic activity by pre-aggregation cells (Gerisch & Hess, 1974 b), the suggestion (Gerisch, 1968) that differentiation into aggregation centers occurs at, and results from, aggregation is now unacceptable.

The role of the aggregation center is strictly connected to the ability to produce and trigger cAMP oscillations in the extracellular medium (Gerisch & Hess, 1974 b; Shaffer, 1975; Roos et al, 1975; Gerisch et al, 1975a). When cells acquire or develop this ability is uncertain; it is clearly a property of at least some of the late-pre-aggregation cells (Gerisch & Hess, 1974 b). It appears also that the first cells to emit cAMP, spontaneously, are unable to sustain the emission for a long time (Durston, 1974 a). It is possible however that these spasmodic signals lead to increased responsiveness and to appearance of additional pacemakers in the population (Durston, 1974 a; Gerisch & Hess, 1974 b). This hypothesis is consistent with the observation that, in an aggregation field, the aggregation centers do not appear at the same instant (Bonner & Hoffman, 1963); particularly in sparse populations, secondary, late developing centers are formed within the boundaries of the original territories.

The rate of emergence of centers appears to be genetically controlled (Durston, 1974 b). It was suggested that the differential secretion of acrasin (cAMP) might be related to the presence of genetically different cells, possibly aneuploids with more than one copy of the genes coding for the control of cAMP production and secretion (Ashworth & Sackin, 1969). The probability of a cell becoming a center was calculated (Durston, 1973) as 1/100 on the basis

that populations of about 100 cells aggregate in 50-100% of the cases (Konijn & Raper, 1961). As well as the absolute cell number in the population, cell density proved to be a critical factor for successful aggregation in these experiments; it is thus uncertain if the data reflect the frequency of active centers or the capacity of the population to differentiate to a stage of aggregation-competence.

Whether a "center" corresponds to a particular cell state (possibly transitory) attainable by every cell in the population at different times remains uncertain. The appearance of new centers on excision of the previous ones (Shaffer, 1962) and the formation of fruiting bodies with as little as 15 cells (Konijn & Raper, 1961) are not evidence enough but support that hypothesis.

It has been shown that cAMP at concentrations of 5×10^{-4}M to 5×10^{-3}M favours center formation (Bonner et al, 1969); it is possible that this is a reflection of a delay in the onset of aggregation in these conditions which would cause a postponement in the competition of centers thereby increasing the final number of those that could dominate a territory. cAMP gradients inhibit, even at concentrations which otherwise would stimulate their initiation, the formation of new centers or even those already formed (Bonner et al, 1969).

It was reported (Ennis & Sussman, 1958) that the appearance of centers was affected by a factor secreted by the myxamoebae; Shaffer (1962) discounted the possibility of this factor being again cAMP by arguing that even aggregation deficient mutants, which are able to respond to artifically introduced aggregates, produced the factor. However this argument ignores the possibility that the mutants might be affected in the mechanism of autonomous signalling though secretion

of cAMP was not totally prevented.

The designation "aggregation center" is also applied to the central cluster of cells resulting from the progress of aggregation. It is questionable, in the hypothesis of a single-cell initiator, if at this stage aggregation is still controlled by the same initial cell.

2-3. The cAMP-signal.

2-3-1. The signal is periodic. The concept that aggregation centers emit pulsatile signals derived from the observation of periodic outward propagating waves in fields of aggregating cells (Arndt, 1937; Shaffer, 1957 a). These waves of movement can be seen in time-lapse films (Arndt, 1929 and Bonner, 1944, 1960 in Shaffer, 1962; Gerisch, 1965; Alcantara & Monk, 1974) and are reflected in early patterns of aggregation amenable to direct observation (Gerisch, 1965; Alcantara & Monk, 1974). However the observed pulsatile response might depend on properties of the reacting cells (e.g. periodicity of refractoriness to a continuous signal) rather than on the periodicity of the signal itself. Shaffer (1962) reported the first strong evidence that the pulsatile response was coupled to an oscillatory activity of the centers of aggregation: aggregating cells can respond alternately to separate centers of aggregation. Robertson et al (1972_a) were able to induce wave patterns centered on artificial, pulsatile sources of cAMP thus adding to the evidence for pulsatile centers; apparently (personal communication) continuous sources of cAMP elicit chemotaxis within a limited range but no propagation of waves can be observed. Gerisch (1968) described a mutant (ap 66) whose aggregation centers induced continuous movement in responding wild-type cells. The author attributed this activity to a chemotactic factor (CF II) operating during aggregation. The non-pulsatile movement towards the mutated

centers implies an inherent ability of responding cells for continuous movement. This property has been confirmed by Durston (1974 a) and Alcantara & Monk (1974). The final evidence for cAMP being released spontaneously and periodically is from the work of Gerisch and collaborators (Gerisch et al, 1974 a, b; Roos et al, 1975) who showed that the periodic chemotactic responses occurring in cell suspensions are related to the periodic release of cAMP into the medium; this oscillatory activity may be triggered by artificial cAMP pulses but not by continuous flow of equivalent amounts of cAMP. The periodic release of cAMP appears to be related to oscillations in the intracellular concentration of cAMP which varies by a factor of 10 during the period of oscillation (peak value=2×10^{-5}M cAMP; Gerisch et al, 1975 a).

Shaffer (1957 a) suggested that the process of modulation of secretion and response to acrasin might be one of the bases for species-specifity of the signal. Since then measurements of the signal frequency (Gerisch, 1968; Durston, 1974 a; Alcantara & Monk, 1974) revealed that even in a single species (D.discoideum) the period of signalling varies, decreasing rapidly during early aggregation from about 10 minutes to 5 minutes; at late aggregation the period is close to 2.5 minutes. Durston (1974 a) proposed that the 5-minute-period corresponds to the period of internal oscillation in the autonomous center and that the frequency of the propagated signals is limited ("gated") by a progressively decreasing refractory period for signal relay in the responding population. This would explain his observation of a bimodal distribution of signal intervals with amplitudes of approximately 10 and 5 minutes. The author suggested that after the 5-minute-period the aggregate is dominated by the recently formed tip (assumed to be a continuous source of signal) to which the cells respond

either with a continuous movement or with a periodicity depending on their refractory period for relay at the moment (about 2.5 minutes). This would mean that the tip and the aggregation center are sources of attractant of a quite different nature; this hypothesis has the added drawback of conflicting with the observation (Robertson et al, 1972 b; Durston, 1974 a) that the subsequent morphogenetic movements in migration and culmination, controlled by the tip, are again periodic. The "gating" effect is still controversial as some aggregation territories exhibit instead a continuous decrease in the signal intervals and no explanation was offered for signal periods of more than 2.5 minutes but less than 5 minutes; in that case the pacemaker could not have been continuous and the period of oscillation was less than 5 minutes, which contradicts the postulated minimal period of oscillation. Because of the prolonged movement response to a single wave front (100 sec) it is difficult to predict whether, during late development, the signal becomes continuous or not but it is conceivable that both the period of oscillation and the refractory period for relay decrease during aggregation. The observation that some pacemakers have signal periods corresponding to multiples of 5 minutes suggested to Durston (1974 a) that periodicity and signal secretion may depend on physiologically separate mechanisms. In mutants (Fr 17) the signal may become intermittent and aperiodic (Durston 1974 b).

The possibility that the cAMP signalling mechanism is utilised in late morphogenetic movements and the implications this might have in the determination of cell position during differentiation are questions open to future investigation.

2-3-2. The cAMP oscillator. The concept of a cellular oscillator was introduced by Goodwin and Cohen (1969) in their model of positional

information in morphogenetic fields. Robertson (1972) adapted the theory to the particular case of aggregation in Dictyostelium discoideum.

Before the striking evidence provided by the measurements of intracellular cAMP concentrations during an oscillation cycle (Gerisch et al, 1975 a) an indication of the existence of internal oscillations in D.discoideum had already been obtained from the observation that the redox state of cytochrome b oscillated simultaneously with the chemotactic activity in cell suspensions (Gerisch & Hess, 1974 b). However the nature of the internal cAMP oscillator as well as its role in the different stages of the life cycle of this organism are still to be clarified. Rossomando & Sussman (1973) suggested that the observed cross activation of ATP-phosphohydrolase and adenyl cyclase by their end products (5'-AMP and cAMP, respectively) might generate oscillations. Goldbeter (1975) worked out a mathematical model based on that report which supports not only the periodic release of cAMP but all the observations by Gerisch & Hess (1974 b) on the interference of artificial cAMP signals with the natural oscillator; he suggested that the simultaneous oscillations in the state of reduction of cytochrome b might be related to variation in intracellular ATP concentration connected with the adenyl cyclase system.

It is possible that the cAMP oscillator may be related to the signal-amplifying system operating during aggregation. This system is thought to consist of cAMP-receptors at the cell surface (with high and specific affinity for cAMP), a process of cAMP synthesis and release through the plasma membrane (perhaps by periodic activation of adenyl cyclase at the cell surface) and the inactivation of extracellular cAMP by phosphodiesterase which allows for a new cycle of amplification (Gerisch et al, 1975 c). Gerisch et al, (1975 b) suggested several

models of relationship between the internal cAMP oscillator and the cAMP signals transmitted across the intercellular medium. To accommodate the data from the experiments on artificial signalling of pre-aggregation cells (Gerisch & Hess, 1974 b) the models favoured were those which included the surface cAMP-receptors as an integral part of the machinery of the oscillator or as part of an extracellular loop of the same oscillator. There is strong evidence that the cAMP-receptors are functionally connected to the cAMP oscillator. This is inferred from experiments on the induction of cAMP oscillations and on the induction of phase-shift in spontaneous oscillations by artificial cAMP pulses (Gerisch & Hess, 1974b) Gerisch et al, 1975 c).

Experiments with the V_{12} M_2 strain, in which ePDE proved to be ineffective in disturbing the rate of differentiation towards the aggregation-competence stage (demonstrated to be under the control of cAMP signalling), and the evidence of loss of sensitivity to artificial cAMP pulses by cells in the pre-aggregation sinusoidal phase of oscillation, raised the hypothesis that the stimulatory effects of cAMP pulses on spontaneous oscillations might correspond to a peculiarity of the Ax_2 strain and that the other strains might be mostly independent of an extracellular control (Gerisch et al, 1975 a, b). Our preliminary work on AF (see Results in this thesis) stimulation of differentiation towards aggregation-competence in NC_4 cells (results not shown) do not favour that hypothesis. Moreover, given the short diffusion-time of the signal (Cohen & Robertson, 1971 a) it is questionable if ePDE, even at high activities, may be expected to prevent cAMP signals from being detected. An intracellular intermediate was postulated (Gerisch et al, 1975 b) for the establishment of the connection between

the cAMP-receptors and the internal cAMP-oscillator as there is no evidence that any component of the oscillator itself is directly accessible to extracellular cAMP signals.

It has been argued that mPDE or ePDE are not directly involved in the creation of cAMP oscillations as it could be shown that addition of 5' AMP has no influence in the pattern of the spectrophotometrically detectable oscillations even when applied in pulses (Gerisch et al, 1975 b). We will present evidence, however, that PDE influences the rate of differentiation during pre-aggregation and probably the establishment of cAMP oscillations (Alcântara & Bazill, 1975; this thesis).

Gerisch et al (1974 a, b) showed that the functioning of the oscillator is necessary but not sufficient for the generation of a signal; internal oscillations may occur even in the absence of any chemotactic activity by sensitive cells. This observation favours the model of an internal oscillator which is facultatively connected to the cAMP-receptors and gives some support to the suggestion of Durston (1974_a) that during early aggregation signals are propagated in multiples of a minimal period of 5 minutes (the suggested period of internal oscillation) due to the "gating" effect of a decreasing refractory period in the responding population. Not so easy to explain is the observation that cell populations, where spontaneous cAMP oscillations had been suppressed by previous treatment with inhibitory concentrations of cAMP, showed chemotactic and signal relay activity in response to artificial cAMP signals (Gerisch et al, 1974 b). The work of Mason et al (1971), Chi & Francis (1971) and Maeda & Maeda (1973) suggested that Ca^{2+} ($>10^{-6}$M) is essential in the phenomenon of aggregation. Gerisch et al (1975 c) suggested that Ca^{2+} is a co-factor

of the process of spontaneous signalling and that it does not have any effect in the mechanism of response (cAMP-binding, chemotaxis or signal relay). Ethylenediaminetetra-acetate (EDTA) and ethylene glycol bis (β amino ethyl ether) NN'tetra-ecetic acid (EGTA) decreased the amplitude of the spontaneous cAMP signal (as evaluated by the responses detected spectrophotometrically) until it became undetectable; addition of Ca^{2+} restored the vigour of the signal although it could not cause phase-shift or appreciable changes in signal frequency. EDTA (10^{-2}M) did not affect the response to artificial cAMP pulses. However the data from Mason et al (1971) show that the synthesis or release of cAMP is not affected by low extracellular Ca^{2+} concentration; in fact both were increased in these conditions. It is therefore difficult to reconcile the data available on the effects of Ca^{2+} on aggregation; further investigation is required.

Another point of interest in the mechanism underlying signalling is the observation by Beug et al (1970) that the binding of non-agglutinating univalent antibody fragments (Fab) to the cell surface prevented aggregation for a period of time which depended on the initial concentration of the Fab fragments (antibodies were prepared against aggregation-competent cells). The Fab treated cells maintained the chemotactic responsiveness towards attracting populations but in these conditions cell-streams were never formed indicating that signal-relay, as autonomous signals, was blocked. As Fab fragments of non-immune sera and Fab of anti-slime mould carbohydrate sera did not prevent aggregation, I think that not only "contact sites A" (see section 4) (as suggested by Beug et al) but also autonomous and relay-signals were blocked by Fab-binding. Admittedly more than one type of membrane component might be inactivated by the aggregation-phase specific Fab fragments and so

the implications this finding may have in the understanding of the mechanism of oscillation are not clear. It would be of interest to know if the internal oscillations are maintained in aggregation-specific-Fab-bound cells.

2-3-3. Time course of appearance of cAMP-oscillations. In agitated cell suspensions extracellular cAMP oscillations and chemotactic activity are initiated before the stage of aggregation-competence (Gerisch & Hess, 1974 b); it has been suggested that the cAMP-oscillator is spontaneously activated during interphase by periodic and small pulses of cAMP (Gerisch et al, 1975 c). No spontaneous oscillations have been detected during growth or early pre-aggregation cells in the system used by Gerisch and collaborators. The difference in extinction that is detectable even in aggregation-competent cells is very small (2%) and it may be that earlier, weaker, signals are undetected; on the other hand, if the decrease in absorbance is dependent on a chemotactic response then the development of chemotactic sensitivity will limit the detectability of early oscillations. This hypothesis agrees with the observation that sensitivity to chemotactic signals develops at the time of appearance of the first oscillations. However the absence of oscillations in the redox state of cytochrome b at these early stages may suggest that the cAMP-oscillator, in fact, is not yet functional or that it has been blocked during early interphase. We should remember in this discussion that cAMP is excreted during early interphase (Malkinson & Ashworth, 1973[a]) and, unless we admit at least two different processes for cAMP release, this fact may indicate the presence of cAMP-oscillations at a much earlier stage than what has been assumed.

2-3-4. Is there a feed-back control on cAMP release? Shaffer (1962) reported that despite the gross difference in the size of selected

aggregates the degree of attraction over responding cells was similar. This was the first evidence for a feed-back control on signal release. Further evidence could be derived from the observation that a constant concentration of 10^{-4}M cAMP inhibited cAMP secretion (and possibly synthesis) by aggregation-competent cells (Mason et al, 1971). Rather strangely (if we admit the inhibition of cAMP release) the addition of cAMP in the experiments by Mason et al did not inhibit center formation (small clumps were formed) but inhibited signal-relay (reported by the authors as inhibition of aggregation). The relation this phenomenon may have with the simultaneous induction of Ca^{2+} outflow reported by Chi & Francis (1971) is not clear. The supression of spontaneous oscillations in cAMP release by continuous cAMP flow (Gerisch & Hess, 1974 b; Roos et al, 1975) is clear indication of feed-back inhibition on secretion; internal oscillations of the redox state of cytochrome b are apparently maintained.

There is also evidence that the amplitude of the relayed signal may be determined by the amplitude of the stimulus. In experiments by Gerisch et al (1974 a) the amplitude of the fast and slow spectrophotometric responses to artificial cAMP pulses depended directly on the concentration of the cAMP solution applied. This contrasts with the report (Shaffer, 1975) that cAMP signals differing by 3 orders of magnitude (10^{-6}-10^{-3}M cAMP) induced similar cAMP-amplification responses.

2-3-5. The size and duration of the signal. It has been suggested that chemotaxis and signal relay may require different thresholds of cAMP concentration and that possibly the threshold for signal relay is higher than the threshold for chemotaxis (Cohen & Robertson, 1971 b). Durston (1973) reported that the two threshold values were, respectively, 10^{-6} and 10^{-8}M cAMP. The upper and lower limits of cAMP concentration

eliciting chemotaxis were determined by Konijn (1972) according to a test devised by himself (1965); they proved to be respectively 10^{-5}M and 10^{-9}M. At concentrations above the upper limit cAMP prevent aggregation by either dispersing the aggregates (10^{-3}M cAMP) or by promoting irregular cell clumping (10^{-2}M cAMP).

A mathematical approach to estimating the value of the threshold for signalling was attempted by Cohen & Robertson (1971 a). It was based on Gerisch's data on the velocity of signal propagation in multilayered cell populations (Gerisch, 1965), on the rate of cAMP production determined by Bonner et al (1969) and on their own assumption that the signal was propagated from cell-to-cell (signal range=one cell row). The authors calculated the threshold for signal relay to be 10^{17} molecules/ml (1.6×10^{-4}M), corresponding to an approximate release of 3×10^{9} cAMP molecules per amoeba and per pulse. However these figures have now little significance since it was shown (Alcantara & Monk, 1974; Nanjundiah, 1975) that the signal range is commonly much greater than the average intercellular distance; moreover, it varies with cell density.

More precise data on cAMP secretion are now available (Roos et al, 1975; Shaffer, 1975; Gerisch et al, 1975 c). The pulse size emitted by responding cells has been calculated by previously labelling the population with $\{^{3}H\}$-8-adenine; when stimulated with cold cAMP pulses (5×10^{-8}M cAMP; 1.5×10^{5} cAMP molecules/cell) the response corresponded to the release of about 6×10^{6} cAMP molecules/cell (amplification factor=40). According to Gerisch et al (1975 c) pulses of 3×10^{3} cAMP molecules per cell (10^{-9}M cAMP) are still detectable by the cell machinery involved in chemotaxis and signal-relay. This is possibly evidence that the two responses do not require different thresholds in cAMP concentration but signal relay may well be dependent on different (possibly

steeper) spatial or temporal gradients of cAMP. This might explain why shallow gradients emanating from continuous sources of cAMP, although promoting chemotaxis, fail to induce wave propagation (Alcântara, unpublished observations).

The cAMP signal was assumed to be not only periodic but also instantaneous (Cohen & Robertson, 1971 a; Durston, 1973). The 0.2 second value for signal duration calculated by Cohen & Robertson (1971 a) was based on this assumption but in fact no indication exists yet for a momentaneous release of cAMP. There is experimental evidence that a cAMP pulse with a half-life of 2 seconds is sufficient stimulus for the activation of the cAMP-receptors (Gerisch et al, 1975 b; Malchow et al, 1974) but, obviously, this does not prove that the spontaneous signal is practically instantaneous. The duration of the chemotactic response to a single wave front (100 seconds) has not been satisfactorily explained in view of the assumed short persistence of the signal. The short relay time ($\sim$ 12 seconds; Alcantara & Monk, 1974), much shorter than the duration of movement response, is a further complication to be considered in the interpretation of the persistent orientation of the chemotactic response: the refractory period for change of direction being not longer than 12 seconds (Alcantara & Monk, 1974) we should expect a cell to be immobilised by its own emission of signal and to reverse the orientation of movement in the subsequent relay step. Shaffer (1975) suggested that a sustained emission of cAMP, giving a different duration and profile to the signal, may constitute the answer to this problem. Some evidence for a prolonged cAMP release may be obtained from the experiments by Gerisch et al (1974 a, b) where the slow peak of response to cAMP pulses (supposed to correspond to the signal-relay phase of the response) had a decay time of about one minute while the fast response (chemotaxis elicited

by the instantaneous artificial pulse) presented a decay time of only 10 seconds; during the period of response to a single cAMP pulse the concentration of extracellular cAMP was shown to increase for about 90 sec (Roos et al, 1975; Shaffer, 1975).

Shaffer (1962) suggested that the amplitude of the signal might vary with cell age and with past stimulation. No evidence has yet supported this suggestion.

2-3-6. The perception of the signal.

2-3-6-1. The receptors of cAMP at the cell surface. Malchow et al (1972) suggested that the rapid appearance of the products of hydrolysis of cAMP in the extracellular medium (< 5 seconds) makes improbable any hypothesis of cAMP being sensed by internally situated receptors. This idea is supported by the fact that the concentration of cAMP that has been used in assays of cAMP-binding by aggregating cells is about 1,000-fold smaller than the intracellular cAMP concentration (Malchow & Gerisch, 1974; Malkinson & Ashworth, 1973a). This was confirmed by Moens & Konijn (1974) who showed, by means of autoradiography and scintillation counts on cell extracts, that no appreciable amount of the label supplied as $[^3H]$-cAMP was associated with the cells after a period of stimulation. The discrimination of cAMP-receptors operating in the process of intercellular communication from other cAMP-binding molecules has to be based on their specificity and sensitivity to cAMP as indicated by the chemotaxis assay; furthermore, besides being subject to developmental regulation, these receptors have to prove to be operational in living cells. The observation by Malchow et al (1973 a) that cGMP is as a good substrate for mPDE as cAMP prompted experiments on the kinetics of cAMP-binding to living cells so far rendered impossible by its rapid (< 5 seconds) hydrolysis by that

enzyme. In these experiments (Malchow & Gerisch, 1974) cAMP was protected by an excess of cGMP which delayed cAMP hydrolysis to about 5 minutes. The cAMP-binding sites detected in these conditions fulfilled the above mentioned requirements. They were sensitive to small ($\sim 10^{-8}$M) amounts of cAMP and their interaction with cAMP analogs paralleled the chemotactic activity of the analogues; both 5'AMP and cGMP showed insignificant binding when compared to cAMP. The activity of the cAMP-binding sites was shown to increase 7-fold during interphase and aggregation and, no doubt, they were active in living cells. The soluble cAMP-binding proteins prepared by Malkinson et al (1973 b) from cell homogenates are thus different from Malchow & Gerisch's binding sites because, although these proteins proved to be developmentally controlled (2-fold increase between t_o and t_{18}), they had similar affinities for cAMP and for cGMP.

The nature of the cAMP-receptor(s) participating in cell communication is still unknown. So is the mode of translation of the extracellular message into intracellular ones. The early idea that mPDE might be the chemotactic-cAMP receptor (Malchow et al, 1972) proved to be unfounded: cGMP, which has a high affinity for mPDE, is a weak competitor of cAMP in chemotaxis and cAMP-binding assays; cAMP-S (adenosine-3', 5'-cyclic phosphorothioate) behaves the opposite way by strongly competing for cAMP-receptors while having a low affinity for mPDE (Malchow et al, 1973 a; Konijn, 1972).

ATP-phosphohydrolase, one of the enzymes which have been implicated in the mechanism of intracellular cAMP oscillation (Rossomando & Sussman, 1973) was the second candidate for the role of receptor of extracellular messages. However the cAMP concentrations required to activate the enzyme, in vitro, and to activate the cAMP-receptors,

in vivo, differ by a factor of 10,000 or more; again, ATP-phosphohydrolase being an integral part of the cAMP oscillator (as postulated) if it would also be the cAMP-receptor we might expect shifts in the oscillations of cytochrome b absorbance (due to fluctuations in ATP concentration) whenever the receptors are activated; in fact, this does not occur when small pulses of cAMP are applied (Gerisch & Hess, 1974 b). Thus the functioning of the cAMP oscillator is possibly not strictly dependent on the activation of cAMP-receptors; this is evidence against the identity of cAMP-receptors and ATP-pyrophosphohydrolase (Gerisch et al, 1974 a). "Contact sites A", the cell-surface components involved in specific end-to-end contacts between stream-cells (probably carbohydrate-associated proteins) have also been rejected as possible cAMP-receptors: inactivation of these sites by specific univalent Fab fragments does not prevent cells from responding to natural sources of attractant (Beug et al, 1970).

The cAMP-binding curves obtained in assays at 23° suggested that the cAMP-receptors have negative-cooperativity properties (Gerisch et al, 1975 c). This finding suggested to the authors an interaction of identical or different cAMP-binding sites which would favour the detection of gradients over a broad range of cAMP concentrations.

Chemotaxis and signal amplification may be related to different cAMP-receptors since, as mentioned above, blocking of signal emission by specific Fab fragments does not affect chemotactic activity. Clearly this observation can be explained in a different way by the blockage of a post-reception step in the mechanism of signal-relay; the observed inhibition of spontaneous signalling is in accord with this explanation. Nevertheless the fact that cGMP protects only some of the cAMP-receptors against inhibition by cAMP-S (Gerisch et al, 1975 c) favours the hypothesis of more than one type of cAMP-receptors.

2-3-6-2. Parameters of cAMP-binding. Scatchard plots of cAMP-binding to aggregation-competent Ax_2 cells, when cAMP was applied in presence of an excess of cGMP, indicated the existence of approximately 5×10^5 cAMP-binding sites per cell and a dissociation constant value of about 2×10^{-7}M. The period of half-maximal cAMP-binding was increased, by the protective action of cGMP, from less than 5 seconds to 1-2 minutes (Malchow et al, 1974).

The cAMP-receptors are activated by cAMP pulses of a magnitude as low as 10^{-9}M (Gerisch et al, 1974 a, 1975 b; Roos et al, 1975). This agrees with the lower limit of cAMP concentration stimulatory for chemotaxis as assayed by the Konijn test (Konijn, 1972). The difference between this stimulus and the intracellular level of cAMP (Malkinson & Ashworth, 1973a) is four to five orders of magnitude.

The binding of cAMP to the receptors is transient and terminates in hydrolysis by PDE (Gerisch et al, 1974 a). Given the low Kd value for cAMP-binding and the fact that Dictyostelium cells only respond to spatial or temporal gradients of cAMP (see below) it is reasonable to assume that in conditions of low PDE activity (stationary phase of growth and early interphase) the receptors may be easily saturated by small amounts of secreted cAMP which thus prevent signal perception.

cAMP pulses elicit at least two types of response in a cell suspension, both reflected in differences of absorbance of light over a broad range of wavelengths (usually recorded at 405 nm). The amplitude of the fast response, supposed to correspond to simple chemotaxis, varies with the size of the cAMP pulse, having a half-maximal peak when the stimulus is of the order of 10^4 cAMP molecules per cell (Gerisch et al, 1974 a, b). This variation in the amplitude of the response may indicate a proportionality between the pulse size and the

amplitude of the chemotactic response if the interpretation of the fast response is correct.

No cAMP-binding could be detected in vegetative Ax_2 cells (Malchow & Gerisch, 1974). These authors also showed that in the V_{12} M_2 strain binding is low at early interphase, increasing by a factor of 7 during interphase and specially during aggregation. The activity of mPDE increases by a similar factor but apparently the time-courses of the two activities differ by 1-2 hours.

2-3-6-3. The mechanism of signal perception. The perception of an orienting gradient of cAMP may be visualised as a process associated with direct sensing of the gradient by, for instance, counting cAMP molecules hydrolised along the cell surface (Malchow et al, 1972). This particular case would require the cAMP concentration to be kept below the limit of saturation of mPDE which corresponds to the hydrolysis of 1.2×10^8 cAMP molecules per cell and per minute. In this hypothesis mPDE would be an essential part of the cAMP-receptor complex.

Gerisch and collaborators (1975 b) proposed an alternative mechanism of chemotactic orientation based on the assumption that expanding pseudopods would be particularly sensitive to changes in the number of cAMP molecules bound to their surface and that the continued expansion of a pseudopod would depend on a positive differential of cAMP-binding in time. Evidence for a time-dependent signal came from the finding (Gerisch et al, 1974 b; 1975 a, b) that cells in suspension respond (chemotactically, by amplifying the signal and in the rate of differentiation) to cAMP pulses, even of small amplitude ($\sim 10^{-9}$M), but not to a continuous flow of equivalent amounts of cAMP. Further evidence for the pulsatile nature of the optimal stimulus came from the observation (Gerisch et al, 1974 a, b; 1975 b, c) that the decline in

the response to a cAMP pulse evaluated by the light scattering method is independent of the continued presence of cAMP after the pulse; it is also independent of the initial concentration of the pulse and of the continued binding of cAMP to the receptors (cAMP was protected from hydrolysis by cGMP). The question is thus one of knowing whether a slime mould cell possesses a "memory" mechanism to compare stimuli occurring at different times or whether it is only able to compare simultaneous stimuli at different points of its surface.

The induction of cell adhesion by cAMP in agar plates was reported to depend on the establishment of a spatial gradient of the chemical (Bonner et al, 1969). The conversion of this continuous spatial gradient into a significant temporal gradient as preconised by Gerisch et al in their model of signal perception is however as hard to realise as the direct perception of the gradient along the cell length. On the other hand, it is conceivable that even the short cAMP pulses applied to a cell suspension may be transformed into short-living spatial gradients. It appears thus that the experiments so far conducted do not discriminate between the two possible models of signal perception involving respectively the comparison of signals on a time basis or the integration of signals along the cell-length ($\sim$10 μm). The temporal model has been favoured possibly under the influence of the recent work on the mechanism of chemotaxis in bacteria (see review by Berg, 1975). Some experiments could be devised to help clarifying this issue. For instance if on supplying cells on a solid substratum with cAMP gradients of progressively increasing slopes the cells extended without displacement this would be evidence for the temporal model; any point at the cell surface would be in a condition to detecting a temporal increase in cAMP concentration and no indication

of orientation could result from the experiment. If the cells were able to perceive the gradient we had to conclude that they were capable of comparing signals along the short distance of their length (10 μm).

2-4. Responses to the aggregation-signal.

2-4-1. Chemotaxis. Chemotaxis is the most evident response to the signal of aggregation. The possibility of observing this response prompted first the qualitative and then the quantitative analysis of this process of cell communication. Cell movement constitutes a direct, easily followed sign of that process and for this reason aggregation in Dictyostelium discoideum has constituted an attractive and rewarding field of research in that area which is fundamental in the mechanisms of development. The fact that the cell population is still, in this phase, a population of single and non-growing cells is an additional advantage for the experimentalist.

Chemotaxis in D.discoideum is seen as a sequence of actions including prolonged and oriented extension of pseudopods and contraction of the cell body. Usually only one pseudopod is formed in response to a stimulus and soon it becomes frontal; if more than one pseudopod is extruded the two or more fronts will compete for domination. Chemotaxis terminates by cell relaxation and withdrawal of pseudopods.

Since cAMP was discovered to be the attractant used by D.discoideum in its self-organized process of aggregation, attempts were made to define, experimentally, the physiological concentrations of the attractant by determining the threshold and upper limit consentaneous with chemotaxis. As mentioned above, Konijn (1972) showed that the chemotactic signal ranged from 10^{-9} to 10^{-4} M cAMP; these values were determined by stimulating spot-populations of sensitive cells deposited

on agar with closely placed drops of cAMP solutions of known concentration; the test was positive if the artificial source of attractant was able to orient the responding population. Similar values of the stimulating range of cAMP concentrations may be derived from the experiments by Gerisch et al (1974 b); in this case the chemotactic response was detected as a decrease in optical density in population of cells suspended in buffer when stimulated with cAMP pulses of known amplitude.

Chemotaxis may thus be elicited both by continuous cAMP sources, provided a spatial gradient is established (even of a minimal slope; case of 10^{-9} M cAMP source in the Konijn test) or by periodic sources. Inhibition of chemotaxis seems to be related to sustained and high concentrations of cAMP.

To account for the continued orientation of myxamoebae towards aggregation centers even when the signal is being propagated outwards it has been assumed that they become refractory to re-orientation sometime after being signalled (Shaffer, 1962). No direct measurement supported this hypothesis. In this thesis and also Alcantara & Monk (1974) we show that the refractory period for chemotaxis, if it exists, does not exceed 12 seconds; Gerisch et al (1975 c) suggested that it is less than 5 seconds. This, as pointed out (section 2-3-5) asks for a re-evaluation of the profile and duration of the cAMP-signal (see discussion in "Results").

The chemotactic response evoked by a single signal front lasts for 100 seconds during which the activated cells move approximately 20 μm inwards; the direction of movement appears to be perpendicular to the threshold concentration contour which first touches the cell membrane (Cohen & Robertson, 1972).

The mechanism of amoeboid movement is still a subject of controversy as evidence has accumulated for (Shaffer, 1963; 1965; 1968) and against (Garrod & Wolpert, 1968) an extensive turn-over of plasma membrane during that process. It is thus unclear whether slime mould cells move by an intensive and localised extrusion of protoplasm accompanied by synthesis of new plasma membrane or if the extrusion is made possible by the elastic or plastic properties of a relatively stable cell surface.

It has been argued that the myxamoebae become polar with respect to production of pseudopods and distribution of cAMP-receptors at the stage of aggregation (Bonner, 1950; Cohen & Robertson, 1972). This idea was contradicted by the observations of Shaffer (1962) and by the experiments of Gerisch et al (1975 c); in this experiments aggregation-competent cells were able to produce new pseudopods at any point of their surface in intervals of about 5 seconds when stimulated by cAMP released locally from microcapillaries. The pseudopods were produced either sequentially or simultaneously. These results agree with the finding that cAMP-receptors are evenly distributed at the cell surface (Gerisch et al, 1975 b). My own observations of cell behaviour in aggregation fields also contradict the idea of fixed cell polarity.

A study of the chemotactic response to a single wave front (Alcântara, unpublished work) has indicated that only after about 12 seconds of continuous pseudopod extrusion does the cell move as whole by retracting the cell body. The cell speed is maximal ($\sim$ 0.5 µm/sec) for the first few seconds and drops then slowly to about 0.1 µm/sec. This proves that the attractant is also a rate substance (i.e. besides its effect on orientation it increases the rate of movement) as suggested by Shaffer (1962) and Konijn et al (1968).

The sensitivity

to cAMP increases by a factor of 10^2 during interphase (Bonner et al, 1969); this is paralleled by an increase in the number of cAMP-receptors (7-fold increase) during the same period (Malchow & Gerisch, 1974). Chemotaxis is further potentiated by the increment in cAMP-production (Bonner et al, 1969; Malkinson & Ashworth, 1973a). Chemotactic activity with distinct cell elongation is already detectable in middle interphase cells and is at least partially responsible for the pre-aggregation patterns described by Gerisch (1965).

2-4-2. Signal-relay. Dictyostelium discoideum has the ability to propagate the attracting signal beyond the direct range of influence of the centers of aggregation and this became apparent to several workers since Bonner (1947) estimated the radius of attraction of the center as being approximately 350 µm. Shaffer (1957 a) called the attention to the fact that the much larger aggregation territories occurring in D.discoideum and in the majority of the other species of slime moulds constituted evidence for a mechanism of signal relay. This conclusion had already been drawn from the very formation of streams or cell chains by aggregating cells (Bonner, 1949) as obviously if the attractant emanated exclusively from the center one would expect separate and individual movement of the responding cells in its direction. Bonner (1950) and Gerisch (1965; 1968) derived further support for the concept of relay from the observation of the concentric wave propagation of the aggregation signal. This also made clear that the conduction of the stimulus in D.discoideum does not require cell contacts (Gerisch, 1968). The signal was already known to be associated with a chemical released into the medium (Runyon, 1942) and its simple diffusion from the aggregation center across the whole field would not explain the non-decrement of attractant concentration along the streams as observed by Shaffer (1957 b; 1962) and Runyon (1942). The concept of signal relay was thus firmly supported by indirect evidence but in spite of

Shaffer's suggestion (1957 b) that the gradient of attractant to which the cells responded was confined to a narrow zone no estimate of the width of that zone had been possible. Cohen & Robertson (1971 a) took, arbitrarily, that width as corresponding to the intercellular distance in a cell population and, using the value of signal propagation (43 μm/min) determined by Gerisch (1965) and their estimate of the time of diffusion of cAMP signals over the 10 μm-intercellular distance (corresponding to the close-packing conditions used by Gerisch), they calculated a relay time of 15 seconds. This means that after being signalled a cell takes 15 seconds to emit its own signal. Given the small time required for cAMP diffusion (less than 2 seconds) the relay time is the limiting factor in the velocity of signal propagation. These authors did not explain,however, their own observation of large areas of cells showing an almost simultaneous response to advancing signal fronts. Subsequently, signal relay was directly observed in cell monolayers and it was shown that the range of the relayed signal covers a width of 57 μm which, depending on the density of the cell population, may include more than one cell row (Alcantara & Monk, 1974 and in this thesis). The relay time value determined on the basis of several parameters of aggregation (see Results) is surprisingly similar (∿12 seconds) to the value calculated by Cohen & Robertson: this suggests that in the multilayer system used by Gerisch or in that particular strain (D. discoideum V12) the signal range did not in fact exceed 10 μm; alternatively the signal range was larger than 10 μm and the relay time did not correspond to the calculations of Cohen & Robertson. The finding that the velocity of signal propagation is dependent on the density of the population supports the first hypothesis (Alcantara & Monk, 1974; this thesis).

Further and definitive evidence for signal relay, and evidence that cAMP acts not only as the attractant but also as the transmitter in the propagation of the signal was provided by recent determinations of extracellular cAMP concentrations before and following short, pulse-like stimulation of cell preparations with cAMP. The release of cAMP was estimated either by the protein-binding assay (Gerisch et al, 1974 a; 1975 b) or by pre-labelling the cells with $\{^3H\}$-adenine (Roos et al, 1975; Shaffer, 1975). The output/input ratio (6×10^6/ 1.5×10^5 cAMP molecules per cell) gave an amplification factor of 40 (Roos et al, 1975). This amplification may correspond to a stimulation of cAMP synthesis or to a mere facilitation of the cAMP efflux through the plasma membrane (Gerisch et al, 1975 b).

The work of Gerisch & Hess (1974 b) indicates that the relay time is independent of the amplitude of the signal and that it possibly decreases during development. The amplitude of the amplified signal was found to be of the order of 10^{-6} M cAMP both in spontaneous and artificially induced cAMP oscillations (Gerisch et al, 1975 b; Roos et al, 1975).

The problem of the extended duration of the inward chemotactic response during the outward propagation of the signal has been discussed above (section 2-3-5) and the possibility of a periodic but prolonged release of cAMP was raised as an attractive explanation of the phenomenon. Support for this hypothesis may be gained from the experiments by Roos et al (1975) where cAMP concentration in the medium was shown to increase progressively until 90 seconds after the stimulation of suspended Ax_2 cells with a small pulse of cAMP. The hydrolysis of the cAMP pulse by PDE was shown to occur in less than 5 seconds (Malchow & Gerisch, 1974) and so there is no doubt that in these conditions release of cAMP is not instantaneous. It is inconceivable

that the 90 seconds half-width of the cAMP peak might correspond to accumulation of cAMP related with a wave of signal relay: the constant bubbling of oxygen, thus the rapid mixing, precludes such a long delay in the stimulation. Similar results were obtained in experiments by Shaffer (1975) using the NC_4 strain; in this case the extracellular medium was analysed at 1 minute intervals after pulsing and, in spite of the extense removal of the medium at each minute after stimulation, the maximal concentration of cAMP occurred in the second interval.

When the mechanism of propagation of the signal was conceived as a relay system it became obvious that to keep unisensal transmission of the signal the cells had to become refractory to activation of signal relay some time after being signalled. This concept was advanced by Shaffer (1962) and was adopted by Gerisch (1965; 1968) to explain the formation of territory boundaries in aggregation fields. It is broadly accepted, presently his suggestion, that the boundaries correspond to the meeting place of waves of signal propagation travelling in opposite senses and that the refractoriness for the signal relay prevents the propagation of both waves beyond the meeting zone.

Durston (1974 a) in a detailed study of the periodicity of several types of pacemakers concluded that the refractory period for signal relay decreases, possibly steadily, from early aggregation until the stage when the aggregate is controlled by a tip and that this was the underlying reason for the stepwise decrease in the apparent signal period.

Estimates of the refractory period for relay were obtained from experiments where slime mould populations in distinct stages of development were stimulated in a pulser apparatus devised by Robertson and collaborators (1972a): a cAMP solution of known concentration was released electrophoretically from a microcapillary in conditions where

both the size and the frequency of the signal were adjustable. Continuous release of cAMP inhibited signal relay (Durston, personal communication); signals at periods smaller than the refractory period were "gated" and propagation was initiated only at the smallest interval exceeding the refractory period (Durston, 1974 a). These estimates showed that the refractory period decreased from about 6 minutes in early aggregation to 2.5 minutes in the late stages of aggregation giving thus support to the suggestion of Durston. However, experiments on artificial signalling of pre-aggregation populations (Gerisch & Hess, 1974b) do not support the high values of the refractory period reported by Durston. cAMP pulses at 5 minute intervals were not "gated" and the cells only showed a decreased response (light scattering method of evaluation) to a second cAMP pulse during the first half of the interval, i.e. for the first 2.5 minutes. Signalling in the second half of the interval elicited precocious cAMP secretion (this property is obviously fundamental for the achievement of synchrony in the process of aggregation). Durston could not explain the continuous decrease in the period of the signal observed in some of the aggregations. The length of the refractory period for signal relay is therefore not yet clearly established.

The molecular basis of the refractory period is still unknown but obviously it has to be connected either with a period of reduced activity of the cAMP-receptors or with decreased secretory activity. Gerisch et al (1975 c) suggested that the refractory period might be associated with a process of adaptation of the cAMP-receptors that can be observed in conditions of pre-incubation with an excess of cGMP or/and cAMP-S: the binding of cAMP is observed to decline with the increase in time of pre-incubation (up to about 2 minutes). In the assay cAMP was protected from hydrolysis by cGMP and by the low PDE

activity of the mutant (wag 6) used. The evidence is however not well established as the decreased binding might be related, as indicated by the authors, to competition with cAMP secreted during the assay. Goldbeter (1975) proposed an attractive alternative according to which the differential sensitivity of the adenyl cyclase oscillator to cAMP during the period of oscillation, as seen in his mathematical model, was the basic feature of refractoriness.

Durston (1973) indicated a cAMP concentration of 10^{-6}M as the minimal concentration capable of evoking a relay response. No experimental support for this figure was presented. This would agree with the early suggestion by Shaffer (1962) that chemotaxis and signal relay might require different thresholds in the concentration of the attractant. However from the set of experiments conducted by Gerisch and collaborators (Gerisch et al, 1974 b; Gerisch et al, 1975a; Roos et al, 1975) it seems reasonable to conclude that not only is the threshold value for signal relay considerably smaller than the value reported by Durston (10^{-9}M compared to 10^{-6}M, cAMP) but also that it is not significantly different from the threshold for chemotaxis. If the signals capable of eliciting one or other of these responses are different, the difference probably lies not in the absolute concentration of the attractant per se but in the profile of the signal as suggested in section 2-3-5. Inhibition of cAMP secretion (Mason et al, 1971) appears to be related to steady concentrations of the attractant as Shaffer (1975) reported that even after 10^{-3}M cAMP pulses secretion of cAMP was not inhibited.

The ability to signal propagation develops late in interphase, later than the increased chemotactic sensitivity to cAMP (Robertson et al, 1972a; Gerisch et al, 1974 a, b). This may either mean that the

two functions, chemotaxis and signal amplification, depend on different cAMP-receptors or that only at a stage does the cell develop the machinery that associates the common signal with the internal amplifier or with the release of pre-synthesised cAMP through the plasma membrane.

There is no evidence to support the hypothesis that signal relay occurs before (as suggested by Robertson et al, 1972a) or after the chemotactic response (Gerisch, 1971). The experiments by Gerisch & Hess (1974 b) suggest that the first response to an artificial cAMP pulse is the chemotactic one and that the cells relay the signal during the chemotactic period. But the indication that the myxamoebae are possibly never refractory to re-orientating signals (Alcantara & Monk, 1974; Gerisch et al, 1975 c) makes that none of the possibilities helps to solve the problem of the sustained directionality of the chemotactic response.

2-4-3. The speed of signal propagation. The velocity of signal propagation in Dictyostelium discoideum was first reported by Shaffer (1962) as 0.5 mm/min (8.3 μm/sec). Later Gerisch (1965; 1968) presented a strikingly different value of 43 μm/min (0.7 μm/sec); it is not clear whether the disparity was due to the conditions of the experiment (densely packed, multilayered preparations) or to strain differences. We have published (Alcantara & Monk, 1974; this thesis) values of velocity varying, according to the density of the population, from 4.70 μm/sec to 6.89 μm/sec. Nanjundiah (1975) confirms this variation but over a broader range: 1.4 to 10 μm/sec.

It has been suggested that the velocity of signal propagation is practically unaffected by the time of diffusion of the signal; Cohen & Robertson (1971 a) calculated that the cAMP signal does not take more than 2 seconds to cover 10 μm and that this was much more rapid than

the value of signal propagation determined by Gerisch. This is however a figure of the same order of magnitude as the values of velocity determined on cell monolayers indicated above but it does not mean that the propagation of the signal is carried out by simple diffusion from the center; Nanjundiah (1975) confirming our finding that the velocity is cell-density-dependent and showing that it increases with the distance to the center of aggregation, dismisses definitely that hypothesis. Our observation of almost simultaneous (<4 sec) chemotactic response over distances of 57 μm (Alcantara & Monk, 1974) suggests that the rate of diffusion of the signal is higher than the value calculated by Cohen & Robertson. Nanjundiah (1975) reports also that the velocity of the signal decreases with the increase in the number of times the center has pulsed but this was possibly due to the simultaneous increase in cell density.

The relay time is one of the limiting factors in the speed of signal propagation, the other being the range of influence of each relayed signal. There is no evidence that the relay time is affected by either the cell age or by the density of the cell population. The results of the experiments by Gerisch & Hess (1974 b) suggest that it is not. One has therefore to admit that the variation of the value of signal velocity reflects a variation in the range of the signal. We have measured directly (Alcantara & Monk, 1974; this thesis) the signal range at densities differing by a factor of 3 (approximately) and we could not detect any significant difference between the two values (57 μm). This is not surprising given that the velocity of signal propagation is only a slow function of the average intercellular distance. Signal ranges cannot be accurately measured in preparations with more extreme values of cell density. Obvious explanations for the

decrease in signal velocity in areas of increased density could be either increased activity of PDE associated with feed-back inhibition of cAMP secretion, or simply an unfavourable ratio between the increase in cAMP output and PDE activity. It has not been proved, however, that PDE can control the signal range. Observations both in favour and against this hypothesis have been reported. Pannbacker & Bravard (1972) showed that the inhibition of ePDE by 1 mM DTT enlarged the range of influence of a source of cAMP. The giant territories of aggregation occurring in ga 86 and ga 88 (V_{12} mutants) (Riedel et al, 1971) might be interpreted as an effect of the reduced ePDE activity in the mutants; however the possibility that the signal range would be increased in these conditions has to be distinguished from another,equally probable, of a decreased rate of appearance of autonomous centers. On the other hand the kinetics of ePDE activity seem to indicate (Nanjundiah, personal communication) that at least this form of PDE is unable to control the spreading of the signal during the short time required for cAMP diffusion. It is unclear what can be the effect of variations in mPDE activity on signal range as it has been shown (Malchow & Gerisch, 1974) that a pulse of cAMP is practically totally hydrolysed by this enzyme in less than 5 seconds.

2-4-4. Aggregation patterns. Several pre-aggregation patterns (feather, ray and mini block) have been described by Gerisch (1965) as reflexions of the functional state of the cells during this early period of development. These patterns are observable only in multilayered preparations and it was suggested that they correspond to the transition from a state of incipient capacity for chemotaxis without pronounced cell elongation to a state of full aggregation competence. It is unclear the role that the presence or absence of

signal relay have in the type of pattern developed; it is more notorious the influence of the relative proportion of aggregation centers in the size of the pattern's motif. Rather surprisingly even t_o cells were able to join more advanced patterns in mixed preparations. In monolayers only one type of pattern can be observed during pre-aggregation and only at its late stages: the "cloud pattern" (Shaffer, 1962; Bonner, 1963). This constituted evidence, confirmed by recent observations (Gerisch & Hess, 1974 b), that already before aggregation some cells secrete substances capable of attracting neighbour cells. Early aggregations, both in multilayers or in monolayers, display a concentric, circular pattern of alternately dark and light zones ("wave pattern"). This was first observed as a dynamic pattern, showing outward propagation of the zones, by Arndt (1937) and Bonner (1944). Shaffer (1962) attempted a theoretical interpretation of this pattern suggesting most of the basic concepts of the mechanism of aggregation as described presently. Gerisch (1965; 1968; 1971), based on his observations of "mini-wave" patterns obtained in multilayered preparations, proposed that the dark bands (whitish in the photograph published) corresponded to vacant zones created by cell displacement in response to a signal front. This raised the hypothesis that the cells showed first a chemotactic response and only after that would the signal be relayed. Both of these propositions proved to be false (Alcantara & Monk, 1974; this thesis) and, in fact, they could not explain the pattern as the 20 μm unitary displacement did not correspond to the width of the dark zone (192 μm).

Apparently even aggregateless mutants (wag 6, wag 11) can form concentric wave patterns (Riedel et al, 1973). This may possibly be due to defects in motility but not in cell elongation as we have shown

(Alcantara & Monk, 1974) that the pattern is directly dependent on the periodic change in cell shape.

"Spirals" are also formed during early aggregation and were described by Gerisch (1971) and Durston (1973; 1974 a, b). It is supposed that they are related to inhomogeneities in the geometry of the aggregation field, to variation of the refractory period for relay in a cell population or to asynchrony in closely situated autonomous centers. They are necessarily preceeded by the occurrence of autonomous, spontaneous pacemakers (which create concentric circular patterns) but, once formed, they are self-sustained through signal relay alone; the signal travels around a loop of variable size and also outwards in the aggregation field. The period of a single spiral depends on the angular velocity (W) of the signal ($P = 2\pi/\omega$; Gerisch, 1971). Spirals are frequently extinguished but if not they will evolve to a minimal period of signalling equal to the refractory period for relay (Durston, 1974 a) concomitantly shortening and closing the central loop. Spirals may be either single or multiple according to the number of wave fronts travelling simultaneously in the loop; pairs of spirals are formed by adjacent loops propagating waves counter-rotationally (Durston, 1973).

The spacing between territories of aggregation was originally attributed to a "spacing substance" secreted by the autonomous centers. This served to justify the uniformity of the size of the territories and thus the constant number of aggregation centers regardless of the value of cell density (Bonner & Dodd, 1962; Bonner & Hoffman, 1963). Gerisch (1965) suggested that the phenomenon resulted from competition within a territory between the numerous loci of periodic signalling by which only that with higher frequency prevail. Boundaries between adjacent territories are straight lines when the respective centers

have similar signal frequency; they are concave towards the one of higher frequency (Gerisch, 1971).

Domination by the first centers is also ensured by the Doppler effect resulting from the inward movement of the responding cells; these cells will be signalled, in fact, at a frequency slightly higher than that of the autonomous center (Cohen & Robertson, 1971 b).

Waves and spirals evolve to the "star pattern" where cell chains of increasing width converge into the aggregation center. The absence of these chains or "streams" in an aggregation field may reflect the blockage of signal relay, the lack of blockage of specific contact sites at the cell surface (contact sites A) or, possibly, as already pointed out, defective motility. Robertson (1972) reported that waves are also propagated along streams; I always found it extremely difficult to follow signal propagation in stream cells, which remain elongated, because of the end-to-end contacts, for most of the already short signal period. Shaffer (1957 b) suggested that the integrity of the streams is preserved, in areas where they move close to each other, thus in conditions where they might be affected by multidirectional stimulation, by a mechanism of "contact-following" which limits and directs the individual cell movement within the stream.

Aggregation is terminated when a hemispherical aggregate is formed; this will continue the course of development when provided with an air-water interface (Gerisch, 1968).

2-5. Factors of aggregation.

2-5-1. Environmental factors. The environment exerts its influence on aggregation both chemically and physically and in ways that can either accelerate, retard or inhibit development. I will consider several elements separately:

Salts. Aggregation is delayed when, after growth, the cells are suspended in water instead of salt solutions (Konijn & Raper, 1961; Konijn, 1972; Malkinson & Ashworth, 1973a). If the myxamoebae are submitted to centrifugation in water even when already aggregating the development will be re-initiated as at t_o (Bonner et al, 1955; Bonner, 1967) but this deleterious effect is prevented if centrifugation takes place in isotonic buffer (Gerisch, 1959). Increased ionic strengths (2-5 fold concentrated Bonner's solution - Bonner, 1947) interfere negatively with cell aggregation to the point of complete inhibition (Konijn & Raper, 1961).

pH. Aggregation occurs within a pH range of 4.5 to 7.0 (Raper, 1940; Hirschberg & Rusch, 1950; Konijn & Raper, 1961). Usually aggregation is at pH 6.1.

Calcium. Mason et al (1971) showed that aggregation is inhibited at concentrations of calcium less than 10^{-6}M; this is probably due to the need for calcium in the mechanism of autonomous signalling (Gerisch et al, 1975 c). It was also reported (Malchow & Gerisch, 1974) that 1 mM Ca^{2+} modifies the cAMP-binding curve by delaying the release of cAMP from the cell surface (addition of 1 mM EDTA has the opposite effect) but the relationship this may have with the mechanism of aggregation is yet unknown.

Humidity. Decreased humidity accelerates the process of aggregation (Raper, 1940).

Light. The effects of light in the early and late periods of development were studied by Konijn & Raper (1966). They concluded that continuous illumination or continuous darkness increases the duration of the pre-aggregation period to a similar extent in comparison with

cases where only an initial period of darkness (optimal=8 hours of darkness in an interphase of about 12 hours) was provided; however, the density of fruiting bodies is increased in conditions of continuous illumination and it is unclear if this reflects an altered rate of appearance of centers of aggregation.

Konijn & Raper (1966) reported also that the sphere of attraction of spot-populations aggregating in the light over sensitive populations was of a smaller radius than that of similar cells aggregating in the darkness. The underlying mechanism responsible for this phenomenon is unknown.

Temperature. Data on the effects of the variation in temperature on early development are inconsistent. It was reported (Raper, 1940) that when the temperature is increased from about 22^{o} to about 29^{o} the pseudo-plasmodia break up into smaller aggregates; it appears thus that aggregation is accelerated by increased temperatures; this was contradicted by Konijn (1965) who observed decreased aggregation activity at high temperatures. The effects will presumably depend on the range of temperatures tested. We have observed that, at 7^{o}, the onset of aggregation is delayed from about t_9 to t_{15} when compared to controls at the standard temperature of 22^{o} (Alcantara & Monk, 1974; this thesis).

Substratum. Dictyostelium discoideum develops on a variety of supports such as agar (0.5-2%), Millipore membranes or glass, in conditions preventing dryness. Shaken cell suspensions do not aggregate unless agitation is slow; in this case the cells agglutinate in clumps of variable size (Gerisch, 1968).

2-5-2. Population-dependent factors.

Effects of the conditions of growth. The Ax_2 strain of D.discoideum, when axenically grown, is prevented from initiating development (presumably by the presence of inhibitory substances) until it is washed and suspended in non-nutrient medium (Malkinson & Ashworth, 1973a). These authors also reported that cells harvested in the phase of stationary growth or in the previous phase of exponential growth will develop in comparable times. This contrasts with recent data by Gerisch and collaborators (Gerisch et al, 1975 a) who reported that stationary phase Ax_2 cells appear to be blocked in the course of differentiation to aggregation-competence. This block could be overcome by stimulation with artificial cAMP-pulses but not by a continuous flow of cAMP solutions. Exponential phase cells were reported to differentiate normally and were only slightly accelerated ($\sim$1 hour) by the artificial signalling.

It was shown (Garrod & Ashworth, 1973 b) that glucose-grown cells mixed with cells grown in non-supplemented medium tend to sort out during development and become spores; additionally the fruiting bodies formed only by glucose grown cells are larger and fewer than in control populations and this may imply that the conditions of growth may influence the rate of appearance of aggregation centers.

Weber & Raper (1971) described two mutants which were inhibited from aggregating by what appeared to be a product of bacterial growth; after washing they aggregated normally.

Population density and cell number. These two characteristics of the population exert separate but interconnected effects on the process of aggregation. Populations of 100-150 cells showed, as reported by Konijn & Raper (1961), an efficiency of aggregation of 90% if at

densities of at least 5 x 10^4 cells/cm2; smaller populations (40-50 cells) showed a comparable response when at densities higher than that value. When the population density was dropped to 2 x 10^4 cells/cm2 a minimum of 500 cells per population was required in order to obtain the same efficiency of aggregation; populations of 100 cells at this density did not aggregate.

It is thus hard to define a minimal (critical) value of population density consentaneous with aggregation; the value commonly accepted (Gerisch, 1961; Cohen & Robertson, 1971 a; Robertson et al, 1972.a)is 5 x 10^4 cells/cm2. The nature of the relationship between the two factors (cell number and population density) is unknown.

Other aspects of interest in the effects of cell density are the delay in the onset of aggregation in sparse populations (Konijn, 1968; Alcantara & Monk, 1974; this thesis) and the decrease in the signal range in dense populations (Alcantara & Monk, 1974; Nanjundiah, 1975; this thesis).

The size of the territory of aggregation is constant regardless of the variation in cell density (Bonner & Dodd, 1962; Bonner & Hoffman, 1963).

3. cAMP-phosphodiesterase (PDE) in growth and in early development

3-1. Forms of PDE and kinetics of appearance and accumulation of their activities. Since the discovery that cAMP is a powerful attractant for Dictyostelium discoideum (Konijn et al, 1968; Barkley, 1969) much work has been concentrated on the purification and characterisation of cAMP-phosphodiesterases (PDEs) from cultures of these cells. At first, interest in PDE was directed mainly to defining its role in chemotaxis where it has the obvious function of reducing the signal-to-noise ratio. At present the interest in PDE is extended since evidence is accumulating that cAMP signalling is involved in early and late development (Gerisch et al, 1975 a; Alcântara & Bazill, 1975; Bonner, 1970; Bonner et al, 1972; Cohen & Robertson, 1971 a).

The first report of the presence of PDE in cultures of Dictyostelium discoideum was by Chang (1968). The enzyme was described as an extracellular form secreted into the medium during growth; it showed a Km of 2×10^{-3}M cAMP and a molecular weight of roughly 300,000 daltons. A cell-bound form was also reported but with a much lower activity. Chassy et al (1969 a) showed that the ePDE was not specific for cAMP; the Km and Vmax values determined by the hydrolysis of several cyclic nucleotides were similar to those found for cAMP. Another form of PDE was described by Pannbacker & Bravard (1970, 1972). This had a lower Km value (1.5×10^{-5}M cAMP) and was found both in the extracellular medium and in the membrane-fraction of the cell homogenate. Chassy (1972) separated two forms of ePDE by ion exchange chromatography and gel filtration. The high Km form was found to have a molecular weight of 132,000, approximately double the molecular weight of the low molecular weight form (65,000 daltons). The reason for the discrepancy in the values of the molecular weight presented by Chang and Chassy is not known; both estimates were according to elution after gel filtration

on agarose columns.

The enzyme described by Riedel et al (1972) and Gerisch et al (1972), with a molecular weight of 60,000 daltons showed an even lower Km (4 x 10^{-6}M cAMP). The low cAMP concentrations found in physiological conditions (up to 10^{-5}M, Malchow et al, 1975 a), suggest that the low Km PDE must be the relevant one in the control of development. Whether or not the Km forms of ePDE correspond to altered forms of one same type of molecule or to different enzymes is not yet clear. Chassy (1972) found that the two forms were interconvertible both spontaneously and by chemical means. Both activities are pH dependent with an optimum at pH 7.5. In contrast with the high Km form, the low Km ePDE is markedly specific for cAMP compared to other cyclic nucleotides (Riedel et al 1972).

The activity of a membrane-bound form of PDE (mPDE) was investigated by Malchow et al (1972; 1973 a, b; 1975 a). This proved to be an enzyme with non-linear kinetics (interaction coefficient less than one), the pseudo-Michaelian constants ranging from 2 x 10^{-7} to 3 x 10^{-5}M cAMP, depending on the range of substrate concentrations. The enzyme has a high affinity for cGMP and cAMP but not for other cyclic nucleotides; cGMP has been successfully used for protection of cAMP in cAMP-binding assays on living cells (Malchow & Gerisch, 1973 c). On gel filtration the solubilized mPDE formed a broad peak of activity from which an average molecular weight of 500,000 daltons was estimated (Malchow et al, 1975 a). The reason for this profile on elution has not been discussed; it would be of interest to know if it corresponds to several degrees of dissociation of a macromolecular complex normally found at the cell surface; such a complex could be responsible for the particular kinetics of the enzyme, and extracellular PDE might be formed from it

on dissociation. Malchow et al (1975 a) proposed that the ePDE (low Km) and the mPDE are partially similar or even identical; this statement was based on the similar substrate specificities (Malchow et al, 1973 a) and on the partial sensitivity to the natural ePDE-inhibitor exhibited by the membrane-bound enzyme when in membrane-fractions or after solubilization. The insensitivity of mPDE to inhibitor in vivo was attributed to masking of the inhibitor-binding site. I think that the partial inhibition of the solubilised mPDE may constitute further indication of the existence of a macromolecular complex in progressive dissociation after solubilisation.

With respect to the kinetics of PDE activity during the life cycle of D.discoideum there are still some controversial points concerning activities at late growth phase and during culmination. Riedel et al (1971) found that the wild-type strain V_{12}, growing in suspensions of E.coli B/r, secreted low Km ePDE, the activity increasing during growth to about 20 units/ml. This contrasts with data of Malkinson & Ashworth (1973 a)who detected a low level of ePDE activity (1.4 units/ml) during growth of Ax_2 cells in axenic medium. The contradiction may result from the difficulty of defining the time of initiation of interphase in the experiments of Riedel et al; it is possible that interphase starts before complete consumption of bacteria (stage defined here as t_o) in conditions where the transition from growth to interphase does not require further treatment (Ax_2 cells, as well as the NC_4 strain, enter a stationary phase between growth and interphase if not washed free of the spent medium). It is therefore plausible that, during growth, ePDE activity is low in all strains. Malkinson & Ashworth (1973a)also reported that this activity was kept constant during growth regardless of the variation in cell density; this is an interesting point but as

yet not clarified. In the growth phase the mPDE activity is kept at a constant, low level of 0.27 units/mg protein (Malkinson & Ashworth, 1973a); this argues against a significant role of PDE during growth.

Pannbacker & Bravard (1972) found that the secretion of PDE in shaken suspensions was particularly intense during the pre-aggregation stage. The activity in the membrane-fraction and in total intracellular-PDE also increased during pre-aggregation and early aggregation and dropped in late aggregation to a level comparable to that observed during the last stages of pre-aggregation. This is evidence that PDE secretion is related to the synthesis or activation of additional enzyme and does not result simply from loss of pre-existing pools; moreover as the high rate of secretion of PDE activity during interphase declines during aggregation when the cell-fraction is enriched in PDE activity, it was concluded that during pre-aggregation the cells develop a mechanism to retain the low Km PDE activity bound to the cell surface.

The kinetics of ePDE activity changes during interphase was closely followed by Malkinson & Ashworth (1973a) and it proved to vary according to the conditions of development. In populations on solid substrata (wet Millipore filters) ePDE activity increased steadily until it reached a plateau of 3.5 units/ml at the stage of middle aggregation (t_{10}), a 2.4-fold increase compared to the growth phase level; the reason why this activity does not decline afterwards, in parallel with the decrease in cell-bound PDE activity, is not known. When cells were kept in suspension in non-nutrient medium and with agitation, ePDE had a peak of activity at an earlier stage (t_2) and of a much greater amplitude; the activity decreased then to values comparable to those observed in preparations on solid substrata, i.e. to about 5% of the peak activities. No data was shown on ePDE activity in shaken suspensions after t_3; if ePDE activity in these conditions raises again at the aggregation stage, with a profile

similar to that described for preparations on solid medium, the t_2 peak may then reflect an artifact due to the agitation, possibly related to an increased rate of ePDE release. This is supported by the work of Malchow et al (1972) who found no significant difference in cell-bound-PDE activity until late interphase when Ax_2 cells were incubated in similar conditions. Alternatively, the t_2 peak may correspond to the active synthesis of PDE in response to an increased rate of secretion of cAMP which has been shown to occur at about t_1 in shaken cell suspensions. The dynamics of cAMP and PDE secretion in different conditions of incubation and at different stages of development suggested that PDE synthesis may be induced by cAMP (Malkinson & Ashworth, 1973_a; Konijn, 1972). These data, in conjunction, favour the hypothesis of active synthesis and secretion of PDE in shaken suspensions at or before t_2.

The inactivation of ePDE during interphase was investigated by Gerisch and collaborators working with several wild-type strains (Riedel & Gerisch, 1971; Riedel et al, 1972; Gerisch et al, 1972; Riedel et al, 1973). These authors proved that this inactivation was due to the release of an inhibitor into the intercellular medium from early interphase (some mutants, as ga 86 and ga 88, were reported to release inhibitor since the growth phase). Toward the end of interphase the rate of production of ePDE activity exceeds the rate of inhibition and PDE activity in the medium increases. In the Ax_2 strain the inhibitor activity peaks at t_4 in shaken suspensions and in conditions where t_{agg} varies from 7-9 hours. In NC_4 and V_{12} M_2 strains the final degree of PDE inhibition varies from 8-60% from one preparation to another, for unknown reasons. The inhibitor appears to be a protein of about 40,000 daltons which is specific for the low Km form of ePDE: it does not inactivate other PDEs or other enzymes secreted

into the intercellular medium by slime mould cells. The inhibitor activity is not affected by treatment at 80° (10 min) which not only inactivates ePDE but also releases the inhibitor from the ePDE-inhibitor complex. This was therefore adopted as a method of preparation of crude inhibitor solutions from the extracellular medium collected during interphase; ePDE deficient mutants (e.g., ga 86) constitute the best sources of inhibitor.

Inhibition is non-competitive and stoichiometric. Experimentally inhibition takes place usually at 35°C although there is low temperature-dependance over the 11°-35°C range; the reaction is almost complete after 15 minutes (Riedel et al, 1972). The species which respond to cAMP as an attractant (D.discoideum, D.mucuroides, D.purpureum and D.rosarium) secrete both PDE and PDE-inhibitor (Gerisch et al, 1972; Bonner et al, 1972). The inhibitors and ePDEs do cross react at least in some of these species. Species which secrete cAMP but do not respond to it chemotactically may (Polysphondilum violaceum) or may not (D.minutum, P.palidum) release PDE spontaneously to the medium (Bonner et al, 1972). PDE inhibition in P.violaceum is not due to the inhibitor described above. The role to be attributed to ePDE in the course of development will have to take into account that cAMP can induce the synthesis and release of this enzyme (Konijn, 1972). Peaks of PDE activity will probably reflect situations of high rate of cAMP synthesis and secretion, the amplitude of the peak being possibly dependent on the contribution of cell-bound PDE to the hydrolysis of cAMP.

Membrane-bound-PDE activity increases during pre-aggregation. Malkinson & Ashworth (1973a) reported a steady increase in activity up to 4.8 units/mg protein (an approximate 50-fold increase in relation to t_o cells) in cells developing on Millipore filters; this was followed

by a decrease to about 1.5 units/mg protein during the late stages of development (15 x the level at t_o). Malchow et al (1972) working also with the Ax_2 strain but in different conditions of incubation (shaken suspensions) obtained values of cell-bound PDE activity during interphase and aggregation which were 6-10 times higher than those of Malkinson & Ashworth (1973 a). The peak value during aggregation was about 15 times the activity at t_1. Quantitative data on mPDE activity aretherefore still contradictory. More recent work by Malchow & Gerisch (1974) showed a time course of mPDE activity in V_{12} M_2 strain in which activity reached a plateau of 1 unit/mg protein at the aggregation stage and Klein & Brachet (1975) presented values comparable to those of Malkinson & Ashworth. Undoubtedly there is an increase in mPDE activity strictly correlated with development and not merely with the arrest of growth: NC_4 and Ax_2 cells in stationary phase of growth do not show increased cell-bound PDE activity (Malchow et al, 1972).

In view of its situation at the cell surface, Km value, resistance to the slime mould inhibitor and the kinetics of its appearance, it was suggested that the mPDE might be associated to the cAMP-receptor-complex operating during chemotaxis (Malchow et al, 1972). This hypothesis however is contradicted by the finding that cAMP-receptor and mPDE have different affinities for cAMP, cGMP and cAMP-S (adenosine-3', 5'-cyclic-phosphorothioate) and that the period of cAMP-binding to living cells is extended to the same degree as hydrolysis is retarded by an excess of cGMP (Malchow & Gerisch, 1974; Gerisch et al, 1975 c).

3-2. Functions attributed to PDE. Shaffer (1956 a) reported that acrasin, the unidentified slime mould attractant, was degraded presumably by an enzyme present in the intercellular medium. In 1962 he suggested that the acrasinase (later identified as PDE) might be

useful in maintaining the detectability of the signal for chemotaxis, especially in a situation where, as he postulated, there are millions of secondary sources of signal. When two Km forms of ePDE were discovered and identified, it was suggested (Chassy, 1972) that both might be involved in the control of extracellular cAMP concentrations, the high Km form preventing accumulation of cAMP beyond the level inhibitory of chemotaxis and differentiation (10^{-3}M).

The hypothesis that ePDE might be a component of a food-seeking device with capacity for adaptation to a mechanism of aggregation was put forward by Bonner et al (1966). Later it was proposed (Gerisch et al, 1972; Gerisch et al, 1974a; Malchow et al, 1975) that during the growth phase ePDE might stop cAMP signals from passing the extracellular space thus preventing chemotaxis. This idea arose mainly from the observation of an increase in ePDE activity at the late phase of growth. However this increase is not detected in conditions which prevent development from immediately following growth (NC_4 and Ax_2 strains grown in suspension) and it seems reasonable to argue that in the V_{12} strains the development is initiated at an earlier time than that indicated by these authors, i.e. before the time of total consumption of food bacteria. This is plausible, as it is known that cAMP does not affect the growth rate, the cell yield or the capacity of the cells to differentiate when added to growing cultures (Malkinson & Ashworth, 1973a) but it has marked effects when added during interphase (Gerisch & Hess, 1974b; Darmon et al, 1975; Alcântara & Bazill, 1975; this thesis).

The inhibition of ePDE during interphase suggested (Riedel et al, 1971; Riedel et al, 1972; Gerisch et al, 1972; Riedel et al, 1973) that a decrease in PDE activity might be required to allow for the cAMP

concentration in the medium to increase to a level which permitted, or actually initiated, development. This hypothesis is hardly acceptable, because it is not consistent with the increase in ePDE activity in the absence of growth (first period of interphase) and the increase in PDE activity during late interphase at the expense of mPDE (Malchow et al, 1972). Furthermore, aggregation competent mutants of NC_4 (fty 17) and of V_{12} (aggr 75) exhibit high ePDE activities throughout interphase (Riedel et al, 1973). Further evidence against this hypothesis emerges from the finding that a constant flow of cAMP added to interphase cells not only does not promote differentiation but may actually delay development (Gerisch & Hess, 1974b; Darmon et al, 1975; Gerisch et al, 1975 a). These are strong reasons for seeking other interpretations of the phenomenon of ePDE inhibition. Gerisch et al (1972) reported that if cells were kept at 8^{o} during a limited period of time at the end of growth phase the production of the ePDE-inhibitor was suppressed and the onset of aggregation was prevented; the supression of aggregation is not confirmed by our own work (Alcantara & Monk, 1974) although, not surprisingly, the onset of aggregation was delayed by about 8 hours. Malchow et al (1972) pointed out that the aggregation deficient and inhibitorless V12 mutant aggr 50 secretes large amounts of PDE into the medium, the level of mPDE, in contrast with the wild-type, being similar to that of the growth phase; thus the mutant is not deficient in the synthesis of PDE but is deficient in its accumulation or activation at the cell surface. If ePDE and mPDE prove to be related (see section above), these observations suggest that ePDE-inhibitor may have a role in the control of activity or in the positioning of mPDE at the cell surface.

The role of ePDE during aggregation is not clear. Pannbacker & Bravard (1972) presented evidence that during chemotaxis ePDE may control

the range of the signal propagated across the aggregation field. They showed that 1mM dithiothreitol (DTT) inhibited ePDE and simultaneously increased the range of influence of an artificial source of cAMP (2.5 x 10^{-6}M) and the sensitivity of the response. This agrees with the parallel occurrence of giant aggregation territories and excessive ePDE-inhibition in the ga 86 and ga 88 mutants of the V_{12} strain of D.discoideum (Riedel et al, 1973). However it has been estimated (Nanjundiah, personal communication), taking into account the parameters of ePDE activity and the rate of diffusion of cAMP, that ePDE is unable to control signal range. The reported increase in the sensitivity of the response after ePDE-inhibition by Pannbacker & Bravard (1972) contrasts with the previous idea that PDE might help chemotaxis by steepening cAMP gradients (Shaffer, 1962; Bonner et al, 1969; Chassy, 1972). However and again according to Nanjundiah (personal communication) the kinetics of ePDE activity are such that it is also ineffective in creating cAMP gradients; its function is probably restricted to degradation of cAMP down to the noise level concentration.

The suggestion of Goidl et al (1972) that inactivation of ePDE by specific antibodies would block cell aggregation was partially contradicted by Riedel et al(1973) who observed that strong ePDE inhibition by the slime mould inhibitor did not impair aggregation. This is not surprising if it is considered that mPDE, which is insensitive to the natural inhibitor, reaches high activities during aggregation; it is conceivable that the anti-ePDE serum used by Goidl et al may also react with mPDE and this might impair aggregation either because this activity may prove to be essential at that stage, or because it interferes with cell contacts.

The fact that the upper limit in the range of cAMP concentrations

eliciting chemotaxis (10^{-9} to 10^{-4}M; Konijn, 1972) corresponds to the cAMP concentration at which the low Km ePDE is saturated favours the hypothesis that this enzyme controls the chemotactic response. However this does not necessarily mean that ePDE is involved in the formation of cAMP gradients; its role may simply derive from the possibility that the chemotactic response requires periodic clearing of cAMP receptors. Malchow et al (1975) showed that mPDE, an enzyme with non-linear kinetics (see section above), takes signficantly smaller times to hydrolyse cAMP to the noise level than a Michaelian enzyme and that this period is also smaller than the period of signalling. mPDE may therefore be relevant in the control of the periodicity of the chemotactic response to cAMP signals emanating from the aggregation center, the sensing of a second signal depending on how fast the first is degraded. A decrease in this periodicity during aggregation has been observed (Gerisch, 1968; Durston, 1974a; Gerisch & Hess, 1974b) and may be related to the progressive increase in the activity of this enzyme (Malchow et al, 1972; Malkinson & Ashworth, 1973; Gerisch et al, 1975 a). It is also thought (Nanjundiah, personal communication) that, due to the kinetics of its activity (increased at low substrate concentrations) mPDE may also be involved in the formation of cAMP gradients near the cell surface.

4. The aggregation-competence state

Cells are said to be aggregation competent if they form specific end-to-end contacts and converge towards centers in streams when they are placed on a solid substratum (Gerisch, 1968). Aggregation-competence is therefore a functional condition of development. Little is known about the intracellular processes leading to this state. More attention has been given to the study of alterations of the cell surface which probably constitute a large part of the whole process.

Gregg (1956) and Sonneborn et al (1964) called attention to the fact that new antigens appear at the cell surface during interphase while some of the old ones are retained. Gregg suggested that these new antigens might function as complementary structures causing specific cell adhesion as hypothesised by Tyler and Weiss for multicellular organisms in general (Tyler, 1947; Weiss, 1947); Gregg proposed also that these new antigens were involved in the coordination of motion during aggregation. The appearance of species-specific antigens during interphase (polysaccharides possibly associated with proteins or lipids) was confirmed by Malchow et al (1967). Subsequently the use of univalent fragments of antibodies directed against vegetative or aggregation-competent cells made it possible to discriminate between old and new antigens on aggregating cells (Beug et al, 1970; 1973 a; 1973 b). Two classes of antibody target sites were identified and they were designated "contact-sites B" (present in growth and aggregation phase cells) and "contact-sites A" (characteristic of aggregation-competent cells). The designations are misleading in some extent because obviously not all the surface antigens necessarily participate in cell contacts but at least some of the antigens specific to aggregation-competent cells proved to be involved in the establishment of the end-to-end contacts which first occur when cell streams

are formed; this was the reason for the designation. Anti-contact-sites B serum (univalent Fab fragments) prevents side-by-side contacts in streams but it does not block aggregation or end-to-end contacts. Similar effects are obtained by addition of 10^{-2}M EDTA (Gerisch, 1968) indicating that the two types of contact-sites differ in their sensitivity to the chelating agent. Blocking of the contact-sites A inhibits spontaneous aggregation but does not impede chemotaxis towards sources of attractant. Thus in addition to cell contacts, some step(s) in signal release is affected by treatment with anti-contact-sites A serum; this finding may be interpreted as meaning that "contact-sites A" are either complex structures related to more than one function or that the designation corresponds to separate and different structures. The number of contact-sites A increases 10-fold during interphase to about 3×10^5/cell. It was calculated, on the basis of this figure and on the size of the Fab fragments ($35 \times 35 \times 60$ A°) compared to the area of the cell surface, that no more than 2% of the cell surface is covered when Fab fragments saturate the surface markers. Aggregation-competent cells therefore share the majority of the surface antigens with growth phase cells (Beug et al, 1973 b). This may be compared with work by Yabuno (1970) who showed that vegetative amoebae are negatively charged and that their electrophoretic mobility decreases gradually during interphase (as well as during the rest of development) because of accumulation of substances (probably lipo-proteins) on the surface; this leads to a decrease of the repulsive electrostatic forces (and possibly to atennuation of contact-inhibition of movement). Garrod & Ashworth (1973 b) related this reduction in electrophoretic mobility to the dramatic increase in non-aggregation-specific cohesiveness (detected by EDTA treatment) which takes place

in the first two hours of interphase; EDTA-resistant cohesiveness, characteristic of differentiated cells, is expressed only from t_2. The decrease in cell speed after $t_{1.5}$ reported by Cohen & Robertson (1972) is probably related to this increase in cohesiveness. One point of interest in aggregation is that the specific end-to-end contacts do not require, according to Gerisch (1968), active metabolism; dinitrophenol-treated cells maintain the ability to form clumps in presence of 10^{-2}M EDTA.

As described above (section 2-3-6) there is a 7-fold increase in the number of cAMP-binding sites at the cell surface (Malchow & Gerisch, 1974) and a substantial increase (15-50 fold) in mPDE activity (Malchow et al, 1972; Malkinson & Ashworth, 1973a) during interphase. Contact-sites A, cAMP-binding sites and mPDE activity have been used as quantifiable markers of aggregation-competent cells (Gerisch et al, 1975 a; 1975 c; Darmon et al, 1975).

Comparison of freeze-fractured aggregation-competent cells with similarly treated vegetative cells by electron microscopy revealed the presence in aggregation-competent cells of particulate structures of increased size (from a minimum of 43 A^o to a maximum of 187 A^o) in the inner surface of the plasma membrane; the increment of the average particle size could be induced by 2 hours of treatment with 10^{-3}M cAMP or 10^{-4}M Ca^{2+} (Gregg & Nesom, 1973).

α-mannosidase and N-acetylglucosaminidase activities (the latter excreted into the medium) increase since t_o but it is still doubtful whether they are to be considered characteristic developmental enzymes or not (Newell, 1971).

The increased production of cAMP during interphase and specially during aggregation is a well established feature of early development (Bonner et al, 1969; Malkinson & Ashworth, 1973a). Cohen & Robertson (1972) suggested that the myxamoebae secrete cAMP first steadily

(period corresponding to the pre-aggregation "cloud-pattern") and only then would they develop the competence to retain cAMP and control its secretion. Gerisch & Hess (1974 b) showed that about three hours before the aggregation-competence stage D.discoideum cells already show periodic chemotactic activity indicative of a periodic release of cAMP. It is questionable whether the functioning of the cAMP oscillator (section 2-3-2) is a characteristic subject to developmental control and therefore only expressed after a period of development as suggested by Gerisch & Hess (1974 b) or whether it is a property of vegetative cells which is amplified during early development (Alcântara & Bazill, 1975; Gross, 1975; this thesis).

The capacity to relay cAMP signals is a characteristic of late pre-aggregation cells (Robertson et al, 1972a; Gerisch & Hess, 1974 b; this thesis) and this is probably one of the factors involved in the activation of pacemakers since it was shown that artificial cAMP signalling induces autonomous oscillations (Gerisch & Hess, 1974 b).

It has been suggested that aggregation-competent cells are polar with respect to signal reception and signal emission (Cohen & Robertson, 1972) or, less precisely, in the capacity to produce pseudopods (Bonner, 1950). Evidence is now against this hypothesis (Shaffer, 1962; Gerisch et al, 1975 c).

5. Trigger(s) and controls of development

Development is triggered by food shortage (Arndt, 1937). It was reported that the conditions of starvation have to be maintained for half an hour for development to be initiated (Cohen & Robertson, 1972). Whether "food" works as a negative control on the development or the lack of it induces a positive control on the expression of a set (or more) of developmental genes is not yet known. Klein (1974) associated the signal of starvation with the intracellular accumulation of guanosine tetraphosphate which forms a sharp peak at t_2; this might control, as in bacteria, metabolic changes resulting in adaptation to nutritionally deficient conditions.

Roos et al (1975) reported that the relative intracellular concentration of guanosine nucleotides increases by a factor of three during interphase and called the attention to the possibility that, as in other eukaryotic cells (Rodbell et al, 1971), they may be involved in the activation of the adenyl cyclase system.

The development of aggregation-competence in D.discoideum was shown to be under control of cAMP oscillations: stationary phase cells, which appeared to be blocked in differentiation, were activated by artificial cAMP pulses (Gerisch et al, 1975 a) the same happening with a group of aggregateless mutants (Darmon et al, 1975). The artificial cAMP pulsing had to be maintained for most of the period of pre-aggregation to be stimulatory. There is thus/a here trigger effect associated with a long term control of development. This control is more clearly manifested by the increase in the rate of differentiation following application of cAMP pulses to normally differentiating cells (Darmon et al, 1975; Gerisch et al, 1975 a). Continuous flow of cAMP does not evoke any stimulation as reported by

the two laboratories. We will show in this thesis (also Alcântara & Bazill, 1975) that ePDE activity added at early interphase shortens this phase to 45-65% of its duration in controls. Our evidence allied to the results of the experiments on continuous cAMP flow invalidates early suggestions that high concentrations of extracellular cAMP were required to trigger or stimulate the shift from the vegetative to the developmental stages (Malchow et al, 1972; Riedel et al, 1971; Riedel et al, 1973). It was suggested that post-aggregation development is also controlled by periodic signalling (Cohen & Robertson, 1971 a). The characteristics of the aggregation-competence stage develop "en block" in response to the stimulation by cAMP-pulses (Gerisch et al 1975 a; 1975 c). This is evidence for an integrated program of differentiation. Furthermore, the stimulation of differentiation by cAMP-pulses and the inhibition of cAMP oscillations caused by supra-threshold, steady concentrations of cAMP (Gerisch & Hess, 1974 b) prove that the process of individual cell differentiation is not independent but rather is controlled by external stimuli which reflect the state of the population as a whole.

It is not clear what effect other mononucleotides as well as glucose, Mg^{2+}, K^{+}, PO_4^{3-} or imidazole containing compounds may have in the rate of differentiation during the early phases of development. Krichevsky & Love (1965), Chassy et al (1969 b) and Krichevsky et al (1969) reported a stimulatory effect of these chemicals on development as evaluated by the number of fruiting bodies formed after a certain period of time. The value of the criterium selected for the evaluation of the stimulating effect is questionable; in addition, the final result may represent the sum of several and opposite effects occurring during the course of development. Cocucci & Sussman (1970) reported

a high level of RNA turn-over during interphase; this is associated with the intracellular accumulation of 2', 3'-mononucleotides (Pannbaker, 1966; Krichevsky & Love, 1968). Krichevsky et al (1969) suggested that the stimulatory activity of supplied mononucleotides in development is due to the counterbalance of the effects of loss of RNA from washed amoebae or to the prevention of that loss.

The establishment of cell contacts has proved to be of prime importance in the process of differentiation during the late stages of development (Newell et al, 1971). The effects of the population density in the early stages are, however, very little known. I show in this thesis that a cell-dependent factor secreted into the medium (ePDE), controls the duration of interphase, but it remains to be investigated whether or not the frequency of random collisions has any influence on the course of interphase. Beug et al (1970) showed that close cell contacts are not essential for aggregation; however there is now clear evidence that the velocity of the signal propagation depends, although not markedly, on cell density (Alcantara & Monk, 1974; Nanjundiah, 1975; this thesis). Areas of close contact may be envisaged as effecting the transfer of small molecules or macromolecules between adjacent cells or just as the process of activation of surface reactions.

It has been shown that differentiation in D.discoideum involves, as in other eukaryotic systems, control of transcription and translation as well as post-translation regulation of enzymatic activities. Polysomes are synthesised "de novo" during interphase (Cocucci & Sussman, 1970) but the significance of this fact is still unclear. For critical reviews on differentiation in D.discoideum see Newell (1971) and Ashworth (1971).

CHAPTER 2

Materials and Methods

1. Strains

Dictyostelium discoideum strain NC4, the axenic strain Ax_2 and the bacterial associate Aerobacter aerogenes; all strains were provided by Dr. J. Ashworth.

2. Media

KK2 buffer contained (g/l water): $K H_2PO_4$,2.25; K_2HPO_4,0.67; $MgSO_4.7H_2O$,0.5; pH 6.1. Standard medium, SM (Sussman, 1951) contained (g/l KK2 buffer): Bactopeptone (Difco), 5; Yeast extract (Difco), 0.5. For SM agar, 20 g Bacto-agar (Difco) were added to 1 l SM broth. After autoclaving (15 lb/in^2, 15 min) sterile glucose was added to SM broth and SM agar to 0.5 % final concentration. Axenic broth, HL5 (Watts & Ashworth, 1970) contained (g/l water): bacteriological peptone (Oxoid 14.3; Yeast extract (Difco), 7.15; $Na_2HPO_4.12\ H_2O$, 1.28; KH_2PO_4, 0.49; D-glucose, 15.4; pH 6.7. Non-nutrient (NN agar contained: Bacto-agar (Difco), 10 g; KK2 buffer, 1.1. Imidazole-HCl buffer contained (g/l water): imidazole, 1.37; $Mg(CH_3COO)_2.4\ H_2O$,0.54; $CaCl_2$, 0.06; pH 7.0. Imidazole-agar contained: Bacto-agar (Difco), 10 g; imidazole-HCl buffer, 1 l.

3. Cell growth

NC4 cells were grown in association with Aerobacter aerogenes. Freshly picked slime mould spores were suspended in a stationary (24 hours old) bacterial culture on SM medium; the density was approximately 7.5×10^6 spores/ml (75 spore-heads/ml). After vigorous swirling 0.2 ml aliquots of the spore suspension were homogeneously spread on each SM plate and incubated at 22°.

Ax_2 cells were grown in HL5 medium in flasks with 10 fold

capacity compared to the volume of culture incubated. The cell suspension was aerated by agitation at 160 rev/min (New Brunswick Scientific gy rotory shaker) and the temperature controlled to 22°.

4. Initiation of development

NC4 plates were harvested 24 hours after plating (exponential phase of growth) by suspending the bacteria and slime mould cells of each plate in 5 ml of cold KK2 buffer; a sterile glass rod was used for gentle detachment of the cells from the agar surface. The NC4 cells were freed of bacteria by four repeated washings in KK2 buffer followed by centrifugation at 400 g for 1 min (MSE Super Minor centrifuge). The cell yield was approximately 10^6 cells/plate. Aliquots of a final resuspension in KK2 buffer were then deposited on NN-agar plates (5 ml. of NN-agar freshly poured on 5 cm x 8 cm boxes; these boxes were manufactured by Arthur Thomas & Co.). In order to obtain populations of homogeneous densities on full plates, 0.5 ml. of cell suspension was spread on the surface of each NN-agar plate and the cells were allowed to settle and disperse for 30 min; the excess liquid was then sucked off and the plates were left open at room temperature for 20 min. Incubation was at 22° except in some cases in which the plates were incubated at 7° overnight and then transferred to 22° for development of aggregation patterns. These patterns developed within one hour after the temperature shift and no qualitative or quantitative differences could be observed between these patterns and the patterns developed after continuous incubation at 22°. Alternatively, 10 µl aliquots of the cell suspension in KK2 buffer were deposited on NN-agar plates which were then incubated at 22°.

Ax_2 cells were harvested in the exponential phase of growth (densities up to 8 x 10^6 cells/ml). They were washed by sedimentation at 260 g for 1 min followed by resuspension in KK2 buffer (2 cycles). The final cell suspension in KK2 buffer, in test or control solutions (see experiments on control of development by AF) was either kept agitated at 160 rev/min in flasks with at least a 10 fold excess capacity, or was spread as thin films on NN-agar plates (0.5 ml cell suspension/plate). "Spot-populations" were prepared by depositing 10 µl aliquots of cell suspension on NN-agar plates. Incubation was at 22° in the light; NN-agar plates were kept in humidity chambers provided with a glass cover.

An alternative way for washing Ax_2 cells was to deposit 10 µl of the growth culture (exponential phase) on an alcohol-washed glass cover-slip and allow the cells to settle for 5 minutes; the preparation was then immersed in 50 ml cold KK2 buffer and the excess liquid drained. The cover-slip was inverted and sealed on a humidity chamber formed by a well drilled in a perspex microscope slide with another glass cover-slip fixed on the bottom. The moisture was supplied by a film of 10% (w/v) gelatin freshly poured into the well. The preparations were incubated at 22°. This technique was only used for some Ax_2 preparations in experiments concerning the propagation of the signal during aggregation; the results were similar to those obtained with NN-agar preparations.

The time of initiation of interphase (t_o) is here defined as the time of final cell resuspension after washing; this followed harvesting by no more than 15 min.

5. Preparation of stock AF solutions

5-1. Culture conditions. Ax_2 cells were grown in 2 litres of HL5 medium to densities of about 5 x 10^6/ml. The cells were harvested and washed twice in cold KK2 buffer by centrifugation at 430 g in a 59563 MSE Mistral 6L head at 4^o for 10 minutes. The cells were finally resuspended at 5-8 x 10^7/ml in KK2 buffer at room temperature and incubated for 10-14 hour in a New Brunswick Scientific G10 gyrotory shaker (160 rev/min) at 22^o and in the light. Two litre Erlenmayer flasks were used for incubation of approximately 200 ml cell suspension. Agitation prevented aggregation but the cells developed full aggregation competence.

Later, when AF solutions with a low content of extracellular-PDE-inhibitor were required, incubation was carried out for 2 hr. only.

5-2. Collecting the AF solution. After the period of incubation, indicated by a subscript of t, the cells were sedimented by centrifugation at 260 g for 1 min in an MSE Super Minor Centrifuge and the supernatant was then re-spun at 10,000 g (Sorval, SM 24 rotor) at 4^o for 20 min.

5-3. Concentrating the AF solution. When required the AF solutions were concentrated in a 90 mm Millipore Hi-flux cell or on a 25 mm stirred filtration cell equipped with PSCA membranes (nominal molecular weight limit = 1,000 daltons). A pressure of 25 psi of oxygen-free nitrogen was applied and filtration with magnetic stirring was carried out at 4^o. Concentration of AF solutions can also be performed by ultrafiltration with membranes of a shorter range of retention. Amicon PM 10 membranes with a nominal retentivity of 10,000 daltons proved to be equally effective for

this purpose.

5-4. Storing AF solutions. The cell-free AF solutions were dispensed in 0.5 ml and 10 ml volumes and rapidly frozen in a dry-ice-acetone mixture. The tubes were then stored at -70°.

6. Fractionation of AF solutions.

6-1. DEAE cellulose chromatography

6-1-1. Preparing the column. 100 g of the anion exchanger Whatman DE 52 (diethylaminoethyl cellulose) were washed in a Buchner funnel with successively 3 l each of 0.5 M NaOH, distilled water, 0.5 M HCl and a final wash in another 3 l of distilled water. The gel was then equilibrated with 0.5 M triethanolamine (TEA)-HCl pH 6.8 buffer for approximately 4 hr. after which the buffer was filtrated away and the gel was washed again with 3 l of distilled water. The ion exchanger was then suspended in 10 mM TEA-HCl pH 6.8 buffer (starting buffer) kept at 4°. For storage, 0.02% (w/v) sodium azide was added.

A 40 cm x 2 cm glass column (Pharmacia Fine Chemicals) was washed and transferred to a cold room (4°C) where all the subsequent operations took place. A funnel with a capacity of 250 ml was tightly adapted to the upper end of the column and the ion exchanger slurry was poured at one time and allowed to pack to a height of 20 cm during a slow drain of the starting buffer. Trapping of air bubbles was avoided. When packing was almost complete the surface layer of the anion exchanger was gently stirred with a pipette and allowed to settle to give a flat surface. The column was washed overnight with 200 ml of starting buffer.

6-1-2. Preparing the sample and loading the column. 55 ml of a 6 fold concentrated t_{10} supernatant (see preparation of AF

solutions) were dialysed for 3 hr against 1 l of starting buffer and overnight against another litre of the same buffer. The final volume after dialysis was 69 ml. From this 2 ml were kept for protein and PDE assays. The other 67 ml, containing 14.8 mg of protein and 535 units of extracellular PDE, were applied on to the column with the help of a peristaltic pump (LKB Varioperpex). The column was washed with 50 ml of starting buffer and the effluent was kept for subsequent PDE and AF assays.

6-1-3. Elution and collection of fractions. The column was eluted with a continuous gradient of 0.0-0.4 M KCl in 10 mM TEA-HCl pH 6.8 buffer. The gradient was obtained by syphoning 120 ml of a 0.4 M KCl in TEA-HCl buffer (flask 1) into 120 ml of this same buffer (flask 2). The containers were of the same size and shape. The homogeneity of the solution was ensured by magnetic stirring in the second flask from which it was withdrawn into the column. The column eluate was pumped to an Ultraviolet Absorptiometer (Uvicord II, LKB) where absorbance readings at 280 nm were recorded. 4 ml fractions were collected by an LKB Ultrorac 7000 fraction collector in a total of 60 fractions. The flow rate during elution was adjusted to 15 ml/hr.

6-1-4. Storing, AF and PDE assays, absorbance at 280 nm. Each fraction was dispensed in two portions: one was assayed for PDE and AF activities immediately after completion of elution and the other was quickly frozen and kept at -70°C for future assays. The initial dialysate and the pre-elution wash were also assayed. Absorption at 280 nm was read again in a Beckman DB G spectrophotometer.

6-2. Filtration on Sephadex G 200

6-2-1. Preparing the column. Sephadex G 200 is a dextran gel with an exclusion limit of 800,000 daltons (protein fractionation range: 5,000-800,000 daltons). Fractionation is according to molecular weight. A 55 cm x 1.5 cm gel column was prepared by first swelling 3.5 g of gel in distilled water at 4°C for 3 days. The gel was then washed and resuspended in 0.017M sodium/potassium phosphage buffer, pH 6.1, after which it was poured into the column (Pharmacia Fine Chemicals). To avoid stratification during packing a 250 ml funnel was fitted to the top of the column and all the gel slurry was poured at one time. Column packing and the subsequent procedure was carried out at 4°C. Care was taken to avoid retention of air bubbles and gel compression was prevented by keeping the operating pressure at 20 cm H_2O. The column was washed overnight with the phosphate buffer.

6-2-2. Loading the column and elution. 2.5 ml of sample (fractions 35-39 of DEAE cellulose chromatography combined in equal parts) containing 0.02% (w/v) Blue Dextran 2000 (Pharmacia Fine Chemicals) and 0.02% (w/v) NaN_3 were loaded on the column after most of the eluant above the gel surface had been allowed to drain. Blue dextran and NaN_3 served as front and end markers in calibration of the column and were eluted, respectively, with 22 ml (V_o) and 72 ml of buffer. The column was eluted with 0.017 M sodium/potassium phosphate buffer pH 6.1, at a flow rate of 5 ml/hr and 65 fractions of 1.2 ml were collected (LKB Ultrorac fraction collector). An LKB Absortiometer was used for localization of protein peaks by absorbance at 280 nm.

6-2-3. Storing and fraction analysis. After completion of elution each fraction was dispensed in 2 volumes: one for immediate use and the other for quick freezing and storing at -70°C. Each fraction was assayed for PDE and AF activities and the absorbance at 280 nm was recorded.

6-3. Disc gel electrophoresis. The procedure was essentially that of Davis' (1964). The method allows for separation of mixtures of protein molecules according to their charge and dimensions (the latter due to the "sieving" effect of the polyacrylamide gel). The sample ions (protein) are introduced near the boundary of two ions (Cl^- and $H_2N.CH.COO^-$) at a pH chosen so that the mobilities of the ions are in the order Cl^->protein>glycine (pH 6.7). When a voltage is applied, the ions separate in order of their mobilities and the protein molecules are concentrated in a narrow band ("stacked") between the slower and faster moving ions. Once this is achieved, the ions are allowed to move into a zone where the pH is such that both glycine and Cl^- ions move faster than proteins. In these conditions a uniform voltage gradient is set up in which the protein molecules move according to their charge and size. A large pore gel may be used for the stacking step, but in order to avoid possible inactivation of the sample during polymerisation of acrylamide, in this work the sample, in 10 mMTris-HCl pH 6.7, was made 40% (w/v) in sucrose and layered directly on the surface of the separating gel.

6-3-1. Preparing and running the gel. 0.5 cm x 11 cm gels were prepared in 12 cm long glass tubes. The tubes were washed with a detergent solution, rinsed in distilled water and dried. The tubes were then stoppered with rubber caps, positioned vertically

and partially filled with a small pore gel solution. This solution contained 7% (w/v) acrylamide, 0.18% (w/v) NN-bis-methylene acrylamide (both from Serva Feinbiochimica, Heidelberg) and 0.375 M Tris-HCl pH 8.8; polymerization occurred after addition of 0.03% (v/v) tetramethylethylenediamine (Serva Feinbiochimica, Heidelberg) and 0.07% (w/v) ammonium persulphate. The gels were poured within 5 min. of mixing; careful filling avoided trapping of air bubbles. Water was gently layered on top of the gel solution to give a flat surface when the gel polymerised. The tubes were left undisturbed for 30 min at room temperature. The water was then drained, the rubber caps removed and the tubes were transferred to the electrophoretic apparatus (Shandon Southern). The reservoirs were first filled with 0.375 M Tris-HCl pH 8.8 for a 20 min pre-run at 1 mA /tube at $4^{\circ}C$; this step was meant to remove ammonium persulphate from the separating gels which could inactivate AF during electrophoresis. The buffer in the reservoirs was then changed for Tris-glycine pH 8.3 (50 mM Tris, 0.384M glycine). The surface of the gels were carefully washed with the new buffer. The sample solution was obtained by a 93 fold concentration of a t_{10} crude supernatant in a Millipore PSAC membrane followed by dialysis against 50 mM Tris-HCl pH 6.7 and addition of 40% sucrose. 100 µl of the sample solution, containing 340 µg protein, was layered on the gel surface, care being taken to avoid loss into the overlaying electrode-buffer in the upper reservoir. 30 µl of 0.1% (w/v) bromophenol blue (Serva Feinbiochimica) was added to the electrode buffer in the upper reservoir for use as a front marker. Electrophoresis was carried out at 0.5 mA/tube for 20 minutes (stacking phase) and at 3 mA/tube for

approximately 2 hours, always at 4°C. The bromophenol blue marker was at about 0.5 cm from the bottom of the gel when electrophoresis was interrupted.

6-3-2. Fixing and staining the gel. The gels were removed from the tubes by gently rimming the tube walls with a syringe needle and by applying a moderate water pressure. The gels were fixed and stained in 0.25% (w/v) Coomassie brilliant blue (Serva Feinbiochimica) in 0.7% (v/v) acetic acid for 2-3 hours. Destaining (in 0.7% (v/v) acetic acid) and staining were carried out at 37°C.

6-3-3. Slicing, solubilisation, PDE and AF assays. Replicas of the stained gels were frozen at -70°C immediately after electrophoresis and were later sliced in 1 mm sections using a gel slicer with 200 1 mm-spaced razor blades. The slicer was kept at low temperature by spreading dry ice on it. Each section was eluted overnight in 0.5 ml 10 mM Triethanolamine-HCl pH 6.8. The eluates were used for PDE and AF assays as described in this thesis.

7. Insolubilisation of AF

7-1. Washing and swelling the Gel. CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals) is an agarose gel (bead form) in which imidocarbonate and carbamate groups are formed after reaction of cyanogen bromide with hydroxyl groups in Sepharose. During coupling stable covalent bonds are established between the imidocarbonate groups in the gel and the amino groups in the protein molecules.

One gram of gel was swollen and washed for 15 min with 250 ml 1 mM HCl. A 25 ml Sinta glass funnel (Gallenkamp), lined with

Whatman 1 filter paper (to help transferring the gel), was used for filtration under vacuum.

7-2. Coupling of AF to Sepharose. Three AF solutions were prepared for coupling to Sepharose 4B; (1) a crude t_{14} supernatant, (2) a DEAE cellulose purified t_{14} supernatant and (3) a crude t_2 supernatant. Aliquots of 4.5 ml of each sample were dialysed overnight at 4°C against 1 l of coupling buffer (0.05 M boric acid-NaOH pH 8.0 containing 0.5 M NaCl; ionic strength approximately equal to 0.5) with a change of buffer at 3 hours. The pH range for best efficiency in the coupling reaction is from pH 8.0 to pH 10.0 . High ionic strength prevents protein to protein binding. After dialysis 4 ml of each solution were recovered; PDE activity in each sample was, respectively: (1) 16 units/ml, (2) 20 units/ml and (3) 64 units/ml. Approximately 0.5 g of swollen gel was taken to couple AF in 3 ml of each dialysed solution. Coupling was carried out at room temperature for 5.5 hrs and at 4°C overnight. In the preparation of inactivated Sepharose (control gel) this step was omitted; all the others were identical.

7-3. Washing and inactivation of Sepharose. Each gel portion was washed under vacuum suction (in separate Sinta glass funnels) with 100 ml coupling buffer and immediately resuspended in 50 ml 1 M ethanolamine pH 8.0. This inactivation of the remaining active groups in the agarose gel by ethanolamine took 2 hours at room temperature, after which protein was removed by six cycles of washing using alternately 0.1 M acetic acid-sodium acetate pH 4.0 containing 1 M NaCl and 0.1 M boric acid - NaOH pH 8.0 containing 1 M NaCl; 300 ml of each buffer were utilised for each gel.

7-4. Coupled PDE activity and gel storage. Each distinct AF gel was suspended in 0.7 ml of KK2 buffer. PDE assays were carried out with agitation to avoid sedimentation of the beads. Final PDE activities in the three suspensions were: (1) 2.5 units/ml, (2) 2.5 units/ml and (3) 4 units/ml, which corresponds to recoveries of PDE activity of respectively (1) 3.6%, (2) 2.9% and (3) 1.4%. NaN_3 (0.02%, w/v) was added to the suspensions before storing at $4^{o}C$.

7-5. Utilisation of Sepharose-bound AF for AF assays. For removal of NaN_3 100 µl aliquots of the stored gel suspensions were centrifuged at 260 g for 30 sec. washed and resuspended in the same volume of KK2 buffer. After resuspension the gel beads were kept dispersed by agitation and 10 µl samples were deposited on NN-agar plates, allowed to settle for 10 min and the supernatant removed by suction (Ependorff pipettes supplied with enlarged-bore tips were used). The AF assay was then carried out (see "AF assay" in this thesis) by placing 10 µl of cell suspension in KK2 buffer on the sedimented beads.

8. AF assay. Except in conditions where the effect of cell density on this assay was being assessed, freshly harvested cells were suspended in sample and control solutions (see "Results") at a density of 5-7 x 10^{6}/ml. Five replicas of 10 µl aliquots were taken from each suspension and deposited on freshly poured NN-agar plates for initiation of development. Imidazole-agar was substituted for NN-agar in the assay for AF activity of rat brain PDE as this enzyme requires Ca^{++} and Mg^{++} and its activity is enhanced by imidazole (Cheung, 1967; Lin et al., 1974).

Incubation was at 22°C, in humidity chambers and in the light. The first signs of cell aggregation, corresponding to the formation of 2-3 cell-streams, were monitored by regular microscopic observation. AF activity is represented by the mean t_{agg} in hours (time of onset of aggregation) for each set of replicas in comparison with the mean t_{agg} in the controls.

9. Preparation of extracellular PDE-inhibitor.

The preparation of PDE-inhibitor followed, essentially, the methods described by Riedel et al. (1972) and Riedel & Gerisch (1971).

9-1. Crude inhibitor solution. The extracellular medium of a cell suspension in KK2 buffer at 10^8 cells/ml incubated at 22° with agitation (160 rev/min; New Brunswick Scientific G 10 gyrotory shaker) was collected after 14 hours of interphase. A 210 ml volume of this cell suspension was spun first at 260 g (MSE High Speed 18-rotor 69179) at 10° for 10 min and then at 12,000 g (Sorval, SS 34 rotor) at 3° for 60 min. Of the clear supernatant 160 ml were dialysed overnight against 1 litre 10 mM triethanolamine HCl pH 6.8, with a change of buffer at 3 hours (the other 50 ml of supernatant were used for a preparation of inhibitor by ammonium sulphate precipitation). Ten millilitres of dialysed supernatant were heated in a water bath at 80° for 10 min and dispensed in 0.5 ml fractions for freezing at -70°. The remaining 150 ml of dialysed supernatant were kept for purification of the inhibitor by DEAE cellulose chromatography.

9-2. Preparation of inhibitor by ammonium sulphate precipitation. Fifty millilitres of cell-free, non dialysed t_{14} supernatant,

prepared as described above, were heated at 80° for 10 min. After cooling, ammonium sulphate was added to 90% saturation (33.1 g solid ammonium sulphate). Precipitation at 7° was carried out overnight and the precipitate was collected by centrifugation at 12,000 g (Sorval, rotor SS 34) at 3° for one hour. The pellet was dissolved in 5 ml 10 mM triethanolamine-HCl pH 6.8 and dialysed overnight against 1.5 l of the same buffer, with a change of buffer at 3 hours. A final volume of 11 ml was recovered, dispensed in 0.5 ml fractions and frozen at -70°. For PDE inhibition this solution was diluted 1:40 in KK2 buffer before being used as stock solutions.

9-3. Partial purification of inhibitor by DEAE cellulose chromatography. 150 ml of dialysed, cell-free t_{14} supernatant (see description above) were applied to a 40 cm x 2.5 cm DEAE cellulose column. The column was eluted with a continuous gradient of 0-1 M KCl in 10 mM triethanolamine-HCl pH 6.8 and 150 four-millilitre fractions were collected (LKB Ultrorac fraction collector) at a flow rate of 40 ml/hr. Fractionation was at 4°. All the collected fractions were dialysed overnight against 4 l of 10 mM triethanolamine-HCl pH 6.8 with magnetic stirring. After adequate dilution the fractions were assayed for PDE-inhibitor activity either directly or following heat treatment (80°, 10 min). PDE-inhibitor formed a broad peak of activity in between fractions 21-76 partially overlapping the peak of PDE activity in fractions 58-67 (PDE activity was assayed before heating). The fractions 40-50 were pooled, dispensed in 0.5 ml volumes and frozen at -70°. The stock solution of inhibitor used for PDE inhibition was a 1:20 dilution in KK2 buffer

of these pooled fractions.

10. PDE inhibition

A t_2 suspension at 5 x 10^7 cells per ml of KK2 buffer was freed of cells by centrifugation at 260 g (MSE Super Minor centrifuge) for 1 min and was clarified by a second centrifugation at 10,000 g (Sorval, SM 24 rotor) at 4^o for 30 min. The supernatant was assayed for PDE activity and used as PDE source in the preparation of series of partially inhibited PDE solutions. For PDE inhibition the method of Riedel et al. (1972) was followed. The PDE-inhibitor solution corresponded to (1) a crude, heated t_{14} supernatant, (2) to an ammonium sulphate precipitate of a t_{14} supernatant or (3) to a t_{14} supernatant partially purified on DEAE cellulose. Aliquots of 0.5 ml of ten different dilutions in KK2 of one of the PDE-inhibitor solutions were added to 0.5 ml volumes of PDE solution; a control where KK2 buffer substituted for the inhibitor solution was included. The PDE-inhibitor mixtures were incubated at 35^o for 25 min and then transferred to an ice-bath. PDE assays were carried out on these solutions.

11. Preparation of rat brain PDE

11-1. Extraction of PDE from rat brain. The method of Thompson and Appleman (1971) was essentially followed. Three rats were anaesthetized with $CHCl_3$, their brains immediately removed (total weight 5.25 g) and mixed with 20 ml 11% (w/v) sucrose. An MSE homogeniser at ¾ maximum speed was used to disrupt the tissues; homogenisation was at 4^oC for 1 min. The apparatus and the sucrose solution were both pre-cooled. After adjustment to pH 6.0 with 1N acetic acid 1.0 ml fractions of the mixture were

treated by sonic oscillation (MSE sonicator, MSE London; setting 5; 2 x 10 sec bursts). The temperature was maintained at 4°C throughout the procedure. The sonicate was spun at 12,000 g (Sorval, SM 24 rotor), at 4°C, for 30 min. Tris-acetate buffer pH 6.0 was added to the supernatant to a final concentration of 50 mM. Final volume was 20 ml.

11-2. Purification of rat brain PDE on BioRad A 1.5. A 105 cm x 2.5 cm BioRad A 1.5 column was prepared on a 120 cm long glass column (Pharmacia Fine Chemicals). This agarose gel has an exclusion limit of approximately 1,500,000 daltons. The column was washed overnight with 50 mM Tris-acetate pH 6.0 and loaded with 20 ml of clarified rat brain homogenate. On elution with Tris-acetate buffer (flow rate 16 ml/hr), at 4°C, 180 four- ml - fractions were collected (LKB Ultrorac, fraction collector) and absorbance at 280 nm was recorded (LKB Ultraviolet Absortiometer).

11-3. PDE assay, Km value and concentrating the PDE solution. After gel filtration of crude rat brain PDE, all fractions were assayed for PDE activity. The reaction mixture was essentially similar to that used for the standard PDE assay: 20 mM imidazole-HCl pH 7.0, 2.5 mM Mg $(CH_3COO)_2.4H_2O$, 0.05 mM $CaCl_2$, 1.4×10^{-5} M cAMP, 10 µg/ml alkaline phosphatase (350 U/mg Boehringer Mannheim) and 10 µg/ml adenosine deaminase (200 U/mg, Boehringer Mannheim). The cAMP concentration was decreased in relation to the standard assay for PDE to improve the distinction between the high and low Km forms of this enzyme. Imidazole-HCl buffer substituted for TEA-HCl buffer as imidazole enhanced rat brain PDE activity and Ca^{++} is reported to be required for maximum PDE activity (Cheung,

1967; Lin et al. 1974). Two PDE peaks were obtained after analysis of the collected fractions: one including fractions 21-35 and the second coinciding with fractions 45-65. The second peak proved to have a Km value of approximately 10^{-6}M cAMP (determined by Dr. George Bazill; the radioactive assay for PDE was used). Fractions within the second (last eluting) peak were pooled and concentrated by ultrafiltration on Diaflo(Amicon) UM2 membranes (nominal retentivity of 1,000 daltons). The concentrate was then dialysed against 20 mM imidazole -HCl pH 7.0, in the cold, for 5 hours, with a change of buffer at 2.5 hours. The dialysate was centrifuged at 12,000 g (Sorval, SM 24 rotor) at 4°, for clarification.

11-4. Storage of rat brain PDE. Rat brain PDE solutions were stored at 4°C as freezing proved to inactivate the enzyme.

12. PDE assays

12-1. Optical assay. For standard PDE assays the method of Michal & Bergmeyer (1970) was followed. A 100 µl volume of the enzyme solution was incubated at 35° with 1.0 ml of a reaction mixture containing 6 x 10^{-5}M cAMP in 0.02 M triethanolamine-HCl (TEA-HCl) pH 7.4, 7 mM $MgCl_2$, 10 µg alkaline phosphatase(350 U/mg) and 10 µg adenosine deaminase (200 U/mg). When required, and in order to get a linear reaction rate, the PDE solution was diluted in TEA-HCl buffer to no more than 35 units/ml. One unit of PDE means the amount of enzyme hydrolysing one nanomole of cAMP per minute at 35°. During the reaction cAMP is converted to 5'AMP at a rate depending on the PDE activity. The combined action of alkaline phosphatase and adenosine deaminase converts 5'AMP to inosine. At 265 nm inosine has an extinction coefficient

which is half of that of cAMP. The change of extinction at 265 nm was read in a Beckman DB G spectrophotometer during incubation at 35° and was continuously recorded in a coupled 10" Beckman recorder, set at a speed of 0.2 in/min. The temperature was allowed to stabilise and the rate of reaction was followed for approximately 15 min. On other occasions (e.g. when many fractions had to be assayed) the absorption at 265 nm was read before and after incubation at 35°; the reaction was stopped by immersion in a water-ice mixture. The PDE activity was calculated on the basis that a 10^{-3}M cAMP solution has an OD_{265}=15. The following formula was used: $2.10^2.V/(15\ v.t)$; V was the final volume (1.1 ml), v was the volume of PDE solution (0.1 ml) and t (in minutes) the time necessary to decrease OD_{265} by 0.1. Alkaline phosphatase and adenosine deaminase were purchased from Boerhinger Mannheim.

12-2. Radioactive assay. For determination of PDE activity in conditions including low substrate concentration (determination of Km values by Dr. George Bazill) the method of Brooker et al. (1968) was followed. Phosphodiesterase, approximately 0.1 unit, was incubated in 0.1 ml 0.02 M imidazole-HCl buffer pH 7.0 or 0.02 M TEA-HCl buffer pH 7.4 both containing 1 µCi/ml [8-H ^{3}] cAMP (specific activity 27.5 Ci/mmole.) plus the required concentration of non-radioactive cAMP. The final cAMP concentrations ranged from 3.2×10^{-7}M to 10^{-3}M. After 10 min incubation the test tubes were transferred to a boiling water bath for three min. After cooling, 1 µg alkaline phosphatase (Boerhinger Mannheim) was added and incubation resumed for 20 min at 37°. One ml of a slurry of washed Dowex 1 x 8,200-400 mesh, Cl^- form, in five volumes

of ethanol was added and the test tubes were shaken for 10 min or more. The contents were then transferred to vials and counted on a Packard Liquid Scintillation Spectrometer in 10 ml of a solution of 16 g PPO, 0.4 g POPOP in 1:1 toluene-Triton x 100.

13. Protein assays

13-1. Reaction with phenol reagent (Lowry et al. 1951). Two solutions were prepared: solution A contained 20 g Na_2CO_3 and 0.2 g sodium/potassium tartrate (crystals) in 1 litre 0.1 N NaOH: solution B was 0.5% (w/v) $CuSO_4.5H_2O$ in water. Solution C was obtained just before use by mixing solutions A and B in a proportion of 50:1, respectively. Appropriate dilutions of the sample protein solution in 50 mM Tris-HCl pH 7.0 were prepared; 0.2 ml of each dilution was added to 1 ml of solution C and both were vigorously mixed. The reaction tubes were left standing for 10 min after which 0.1 ml of a 1:1 dilution in water of phenol reagent (Folin & Ciocalteau reagent) was added to each tube and briefly vortexed. The phenol reagent solution was purchased from Fisher Scientific Co. The reaction was carried out at room temperature for 30 min or more. The blue colour produced after reaction is a result of a biuret reaction of protein with copper ions in alkali followed by reduction of the phosphomolybdic-phosphotungstic (Folin & Ciocalteau) reagent by tyrosine and tryptophan present in proteins. The protein content was calculated by comparison of extinction values at 740 nm with a standard curve constructed in each assay, from absorbance readings (Beckman DB G spectrophotometer) corresponding to five different dilutions of a 200 µg/ml solution of bovine albumin (BDH) in 50 mM Tris-HCl pH 7.0. The range of the protein concentration was 0-50 µg/ml.

13-2. Absorption at 280 nm (Warburg & Christian, 1942). The absorption at 280 nm was measured in a Beckman DB G spectrophotometer in 1 cm wide cuvettes. This method of estimation of protein content was used when a large number of samples were to be assayed (e.g. after fractionation procedures). The absorbance at this wavelength is primarily related to the amount of tyrosine and tryptophan present. For a more accurate calculation of protein concentration this method would require a correction factor (OD_{280}/OD_{260}) for the absorption due to nucleic acids, purins or pyrimidines present.

14. Chemotaxis assay

14-1. Preparation of "spot-populations". Ax_2 cells were suspended in test and control (KK2 buffer) solutions at densities of approximately 7×10^6/ml. Immediately after suspension, 10 µl aliquots of each population were deposited (spot-populations) on freshly-poured NN-agar plates (8 aliquots per plate). Incubation was at 22°C, in humidity chambers and with illumination.

14-2. Challenge with cAMP. 10^{-4} and 10^{-5} M cAMP solutions in KK2 buffer were prepared. At hourly intervals from t_o, two populations of each series were challenged with one of the cAMP solutions; for this, 5 µl (Eppendorf pipettes) cAMP solution was placed at approximately 1 mm from the edge of the test population. Distinct populations were used at different times.

Alternatively, in experiments devised to determine the refractory period for movement during aggregation, cells were allowed to develop full aggregation competence and start aggregation when they were challenged with 2 µl of 10^{-3} M cAMP solution in KK2 buffer, absorbed on/a filter paper (Whatman 1) 3 mm square; this artificial source of cAMP was also placed at approximately 1 mm

from the edge of the test-population.

14-3. Cell responses. Cell responses may be monitored by observation in a plate microscope (Nikon) under 40 x magnification but, for better detection of early responses, 125 x magnification in phase-contrast conditions (Wild M 20 microscope) was preferred. Observations were at ½ hour intervals.

Responses to cAMP may correspond to individual cell displacement with clear orientation toward the artificial source of attractant; it is seen, on the whole, as a common cell orientation within the range of influence of the source. At later stages the previously independently moving cells form stable end-to-end contacts which cause the formation of cell-streams moving in parallel toward the artificial source of attractant.

When cells are challenged at the stage of early aggregation streams are broken up within the range of influence of the source of attractant and the cells reorient themselves in direction of this source either as separate cells or as newly reorganised streams.

15. Cell density.

Cells were counted in 0.1 mm deep improved Neubauer chambers. When necessary the cell density (cells/ml) was adjusted by dilution.

Cell densities per unit area were determined by using calibrated eyepiece graticules. The area of each square in this graticule corresponded to 8.8×10^{-5} cm 2 in the preparation. For estimations of density cells were counted in 25 squares (cells/cm2 (N) = $n \times 10^4/22$; n, number of cells in 25 squares).

16. Intercellular distance

Intercellular distances were determined according to the formula $2/\sqrt{(\pi N)}$ where N is cell density per unit area. In this formula the slime mould field is approximate to a uniform distribution of amoeba in which each cell is equidistant from its neighbours; the field is regarded as a network of contiguous circles centred on individual cells.

17. Optical methods

17-1. Microscope observations and measurements. A Wild M20 microscope was used for phase contrast observations at 125 x magnification. It was equipped with a long working distance condenser (N.A. 0.52) and with a humidity chamber fitting both the slide carrier and the boxes used for preparation of NN-agar plates.

The post-aggregation stages of development were observed under 10-40 x magnification in a Nikon plate microscope.

For direct microscope measurements an eyepiece scale was calibrated against a stage micrometer.

17-2. Photography. A Rada roll film back (6 x 9 cm) and an adaptor cone were fitted on the vertical tube of a trinocular phototube in the microscope. In between the adaptor and the eye-piece a Wild photocell was installed. FP4 (125 ASA) films were used for exposures of 1 sec at a light reading of 30 (Unigalvo meter). The concentric wave pattern in D.discoideum is particularly difficult to photograph. A special photographic assembly was organised by J. Kinross. A form of dark field was obtained by using a Kodak cold light illuminator masked with

black cards leaving a 1 cm wide slit. The light was concentrated by a 110 mm condenser placed at 29.5 cm from the light source and a second, plano-convex, 180 mm diameter condenser placed 24.5 cm above the first. The slime mould preparation, situated on the plane face of 180 mm condenser, was photographed with a Polaroid MPP3 camera fitted with a 4 x 5 in sheet film (Ilford FP4 film, exposure ⅛ sec at f=4.7).

17-3. Films and film analysis. A Bolex H16 Reflex cine camera fitted in a Wild M20 microscope and a Paillard/Wild Variotimer were used for time lapse films. The microscope was supplied with a trinocular phototube Hu allowing for 100/25/0 % observation which introduced a magnification factor of 1.25. The vertical tube, with which the cine camera was associated, was fitted with a 10 x compensating eyepiece. A Wild photocell was attached to this tube when setting of illumination was required, and then removed before the cine camera was correctly placed just above and centred with the eyepiece (centring had been checked beforehand). The cine camera was equipped with a 75 mm 'C' mount lens, set to infinity. The oblique tubes in the phototube, used for direct observation, were fitted with a 10 x compensating eyepiece and with a compensating format-indicating eyepiece; this had been previously calibrated and centred in relation to the cine camera ground screen. The slime mould preparations were placed in the microscope humidity chamber. Fogging in the objective was avoided by a fitted warming sleeve which raised the temperature in the preparation by not more than 0.5^{o}. The light, measured at the microscope eyepiece (beam split = 25%) was set to a reading of 30 in a Unigalvo meter; the preparation was kept

illuminated over the whole period of filming. The Variotimer was set to 0.4 sec.of exposure and to 3.6 sec. of interval between exposures; this gave a film speed of 1 frame/4 sec.

A Litax film analyser provided with speed control (frame by frame; sets of any number of frames; 8, 16 or 24 frames/sec) and with facilities for cell tracing, was utilised.

The films were calibrated by filming a stage micrometer in the same conditions as the slime mould preparations.

CHAPTER 3

Results

1. Propagation of the aggregation signal in Dictyostelium discoideum

1-1. The concentric wave pattern and the velocity of signal propagation.

During the first hour of aggregation the cells of D.discoideum organise sets of concentric rings around the aggregation centers. This pattern has been described by Shaffer (1957a,1962) and by Gerisch (1965). From the observation of this pattern these authors derived velocities of signal propagation of, respectively, 500 µm/min and 43 µm/min; the diameter of each territory of aggregation (each set of rings), as reported by Gerisch, was approximately 1.4 mm.

Fig. 1 illustrates the concentric wave pattern in D.discoideum when the cells were suspended in KK2 buffer at a density of 4×10^6/ml and then spread on NN-agar plates (0.5 ml per plate); the amoebae were allowed to settle and disperse for 30 min, the excess liquid sucked off and the agar surface partially dried by leaving the plates open for 20 min. Incubation was at 22° with illumination. This technique produced regular results provided the NN-agar plates were recently poured. Similar patterns were obtained when the cell density was increased up to 5 fold, the cell density per area increasing from 10^5/cm2 to 5×10^5/cm2. Besides the concentric wave pattern spirals may also be observed. On each plate at this stage the size of the aggregation territories varied; they ranged from 4 mm to 10 mm in diameter. Aggregation started normally with territories up to 70 mm in diameter which would soon break up into smaller areas taken over by new centres.

Microscopical observation (magnification 125 x) of the aggregation field revealed that the light, whitish bands in the pattern were formed by oriented, actively moving cells (movement bands). In disagreement with a previous interpretation of the wave

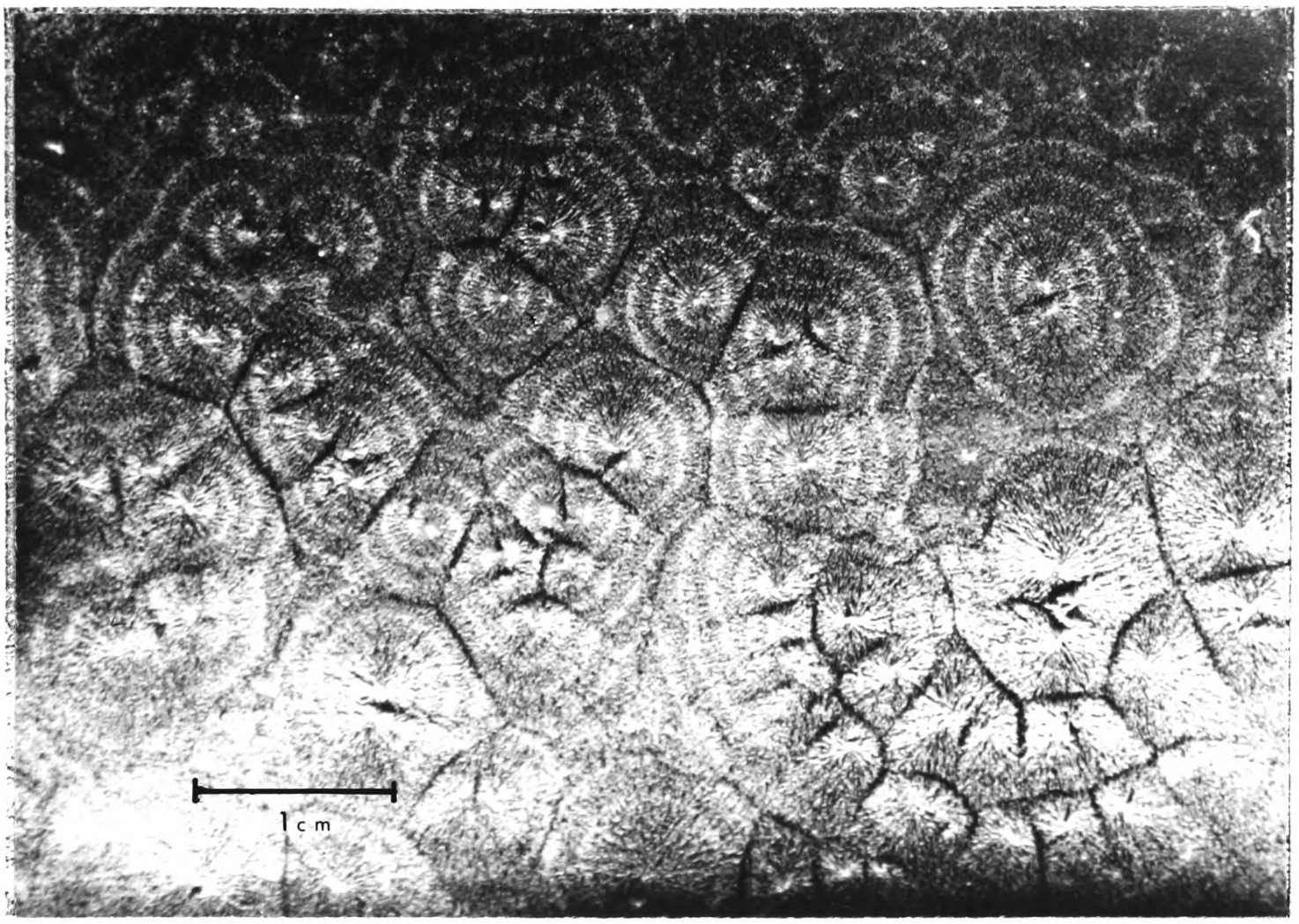

FIGURE 1

The concentric wave pattern in fields of D.discoideum, strain NC4, aggregating on the surface of NN-agar (cell density: 10^5 amoebae/cm2). The light bands are bands of moving cells, each band corresponding to one signal emanating from the respective center of aggregation. The darker bands (interbands) are zones of more or less quiescent cells. Some spiral wave patterns are also present. The photograph (by J. Kinross) was obtained by arranging a form of dark field illumination (as described in Methods).

pattern (Gerisch, 1968), no differences in cell density could be observed within a single territory of aggregation; the darker bands did not correspond to vacant zones as previously postulated but rather were formed by non-oriented, slowly and randomly moving cells of a more or less isodiametric shape (interbands). Fig. 2 illustrates this observation by showing the junction between a interband (IB) and a movement band (MB) in an aggregation field of NC4 at a density of 1.7×10^5 cells/cm2. The visible pattern probably results from differences in light scattering in the two bands due to different cell distribution or to modification in cell shape during active movement.

Detailed observation of aggregation fields and the analysis of time-lapse films (1 frame/4 sec) showed that each movement band was the front of propagation of a signal emanating from the center of aggregation. The signal was smoothly propagated outward while the cells in the innermost region of the movement band slowed down and rounded up after a period of response. It was clear that the MB was greater than the zone of influence of a single signal, as cells within it started responding to a propagating signal at times differing by as much as 100 seconds; this delay could not possibly be attributed to the time required for diffusion of the attractant (diffusion coefficient of cAMP=4×10^{-6} cm2/sec; Cohen & Robertson, 1971a). The movement band was interpreted as representing the distance the signal travelled in the time the cells remained elongated after stimulation. The duration of the movement response to a single signal, corresponding to the interval of time in which the cells remained elongated and moving at increased speed ($^{speed\ average}/_{0.1\ \mu m/sec}$), was measured in time-lapse films; it had a mean

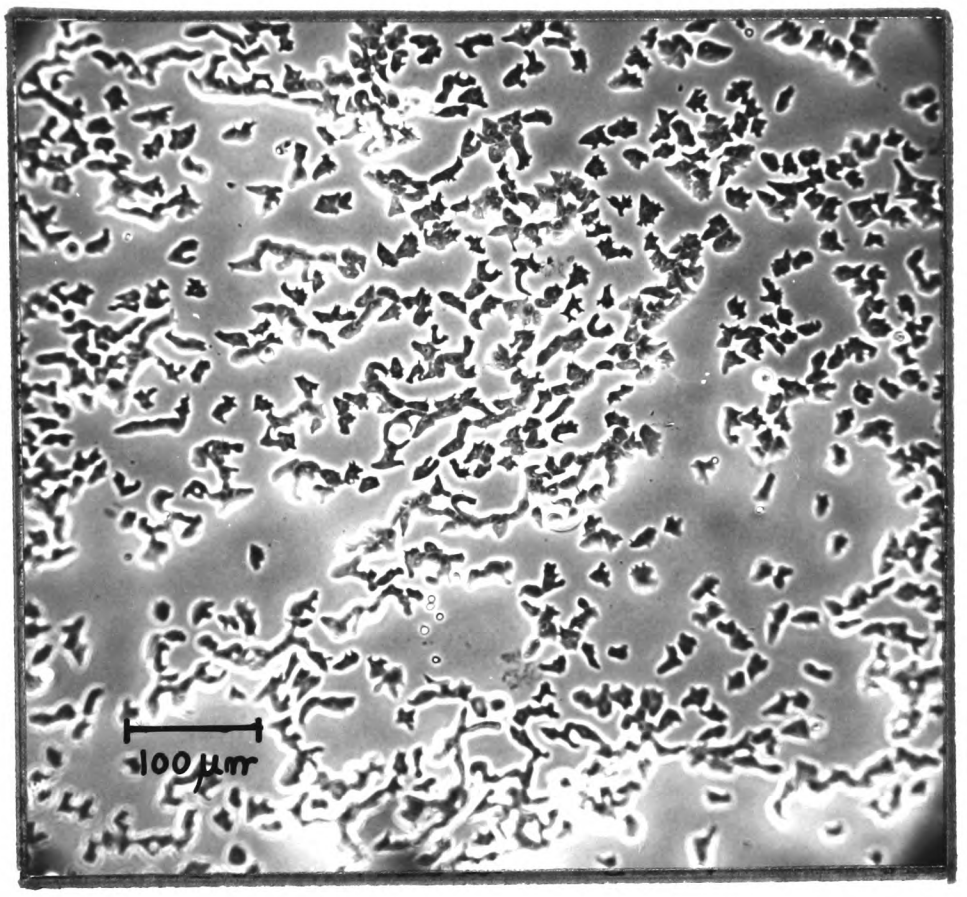

FIGURE 2

The junction between a movement band and an interband in the concentric wave pattern of NC4. Cell density was 1.7×10^5/cm2

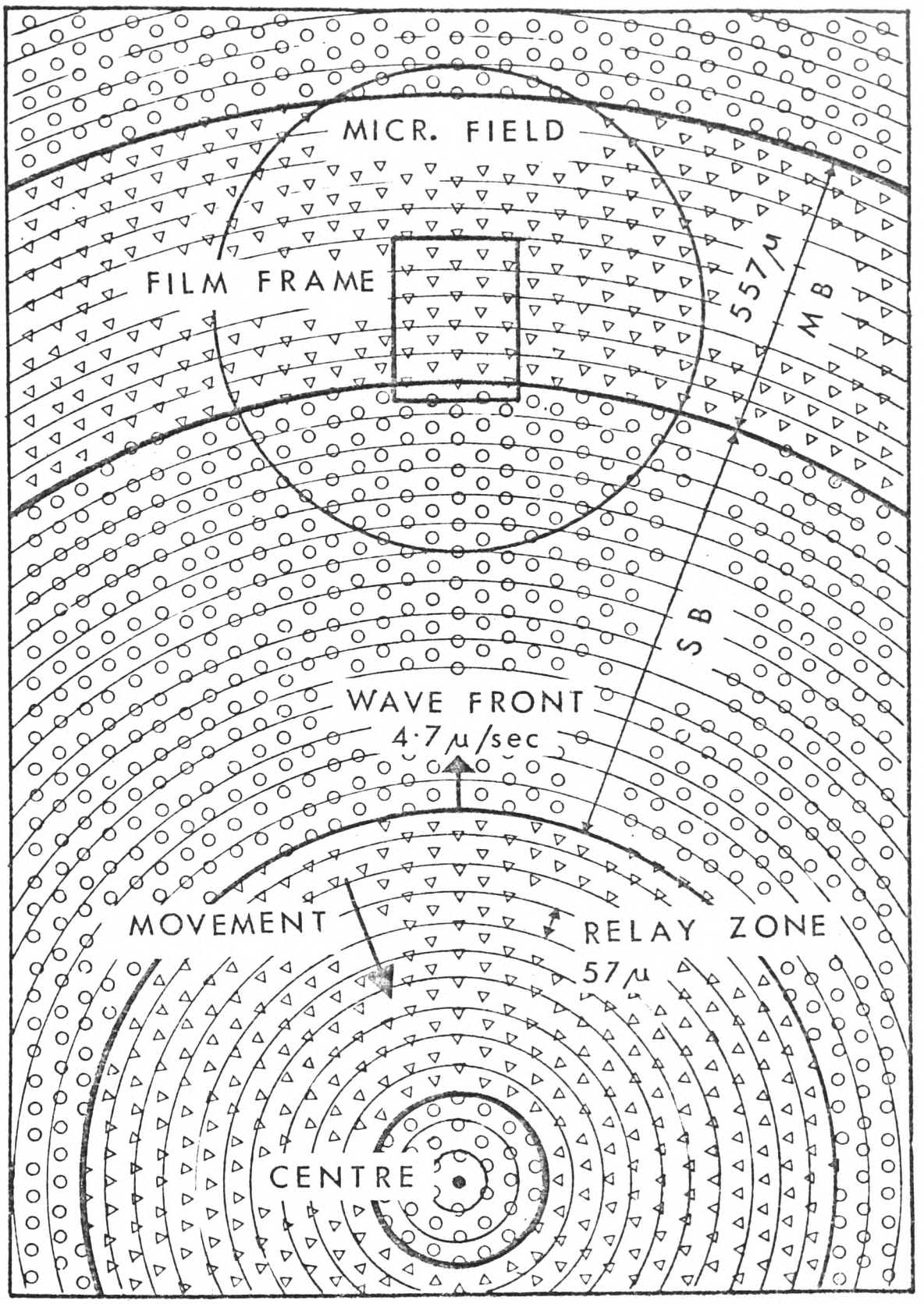

FIGURE 3

Model of signal propagation. The field of amoebae is divided into concentric relay zones. Each relay zone is depicted as encompassing only one or two amoebae. This would be true for sparse aggregation territories. For denser territories see text. Two wave fronts, corresponding to two consecutive signals from the center, are shown propagating outward. The direction of cell movement is inward as shown. The sizes of the microscope field and film frame used to collect the data are indicated, in relation to the size of the aggregation territory as a whole. MB, movement band; IB, inter-band; ▽, moving cell; O, non-moving cell.

value of 99.5 ± 13.7 sec (appendix 1). This agrees with the value (100 sec) presented by Cohen & Robertson (1971b). After disappearance of the chemotactic signal (by diffusion and enzymatic activity) and when the movement response ceased at the inner edge of the movement band, cells rounded up until the wave front corresponding to the next signal reached them. This transition smoothly extended the interbands outward. The width of the interband, according to this interpretation, might be variable and dependent on signal frequency since it is determined by the distance the wave front has propagated during the time elapsing between two successive signals. The wavelength, comprising a movement band plus the adjacent-inner interband, should be proportional to the period of the signal, the width of the movement band being constant. The signal frequency is known to vary during aggregation (Shaffer, 1962; Gerisch, 1971; Durston, 1974a) and so the prediction above could be easily tested. The variation of the signal period was confirmed and the results are shown in Table 1; the period of the signal was estimated by timing individual responses of chosen cells to successive signals in the analysis of time-lapse films taken at a speed of 1 frame/4 sec. The velocity of signal propagation was calculated by three different methods, all based on the interpretation of the structure of the above concentric wave pattern as described/(see Fig. 3): (1) wavelength divided by the period of the corresponding signal, (2) movement band width divided by the duration of movement (100 sec) and (3) distance between any chosen cells divided by the interval of time between responses to the same signal front.

The signal period was measured, here, by directly timing the interval between movements of selected cells in response to

TABLE 1

Strain	Signal Period (sec)	Time after initiation of development (hr)
NC4	420	
	257	
	320	
	327	7½
	277	
	340	
	243	
	413	8½
	208	
	239	9
	129	
	168	
	217	9¾
	163	
Ax_2	580	
	460	8
	428	
	328	
	360	
	340	
	336	9
	332	
	320	

Variation of signal period with time of aggregation. From analysis of time-lapse films of aggregating NC4 and Ax_2 cells, the signal period was measured as the time difference between two successive movement steps of chosen cells in the field, corresponding to two successive wave fronts of movement. For each aggregation stage the period corresponding to successive pulses was measured in the same area of the aggregation territory.

two successive signal fronts. The movement band was easily and rapidly measured under 125 x magnification by using a calibrated microscope eyepiece, the whole movement band fitting in the optical field. The widths of the interbands usually exceeded the microscope optical field. These widths were calculated by rapidly scanning the preparation during an outward movement starting from the corresponding movement band; particular features in the preparation served as reference points in the sequential measurements. The widths of the movement bands varied from 470 µm to 705 µm and the widths of the interbands ranged from 451 µm to 2021 µm. The parameters in Table 3 (distance between chosen cells and interval between responses) were measured in time-lapse films (1 frame/4 sec) analysed at appropriate speed (8 frames/sec and frame by frame). A fair agreement exists between the values of signal velocity expressed in Tables 2 and 3. The velocities of signal propagation which would be obtained by dividing the movement-band widths in Table 2 by the value of movement duration (100 sec) also agree well with the values of velocity listed in Tables 2 and 3 (see also Table 4). The signal velocity varied, depending on cell density, from 3.1 to 8.9 µm/sec (186-534 µm/min). This agreement in the values of signal propagation evaluated from six different parameters constitutes a positive test of the proposals, made above, concerning the mechanism of formation of the wave pattern.

1-2. The relayed signal: signal range and relay time.

It is known that the process of aggregation in D.discoideum involves signal relay (Bonner, 1949; Shaffer, 1957a,1962; Runyon, 1942); this process enables one center of aggregation to encompass areas of up to several centimeters in diameter, though its range of

Variation of wave pattern with signal period in D. discoideum NC4

Cell density* (amoebae/cm²)	Signal period (s)	Movement band† (μm)	Interband (μm)	Wavelength‡ (μm)	Velocity§ (μm/s)
$1{\cdot}25\times10^5$	224	536	780	1316	5·9
(32)	184	489	451	940	5·1
$1{\cdot}5\times10^5$	480	470	1645	2115	4·4
(29)	330	470	1410	1880	5·7
	290	470	705	1175	4·1
	239	470	564	1034	4·3
$1{\cdot}7\times10^5$	300	705	752	1457	4·9
(27)	290	658	658	1316	4·5
	285	658	611	1269	4·5
	275	658	658	1316	4·8
	270	658	611	1269	4·7
	260	611	752	1363	5·2
$2{\cdot}5\times10^5$	600	470	1645	2115	3·5
(22·5)	530	470	1880	2350	4·4
	530	470	1457	1927	3·6
	330	470	940	1410	4·3
	310	470	658	1128	3·6
	285	470	752	1222	4·3
5×10^5	600	470	2021	2491	4·2
(16)	580	470	2021	2491	4·3
	290	470	564	1034	3·6
	275	470	564	1034	3·8

* The intercellular distances, in μm, are given in parentheses.
† The velocity of signal propagation can be calculated by dividing the movement band widths in this column by 100 s, the movement duration (see text).
‡ Sum of the widths of a movement band and the corresponding interband.
§ Derived from wavelength/signal period.

TABLE 2

Velocity of signal propagation determined by measuring the time interval between movement responses of individual cells

Strain	Cell density* (amoebae/cm²)	Distance between chosen cells (μm)	Interval between responses† (s)	Velocity‡ (μm/s)
NC4	5×10^4	143	16(2)	8·9
	(51)	128	16(3)	8·0
		114	16(3)	7·1
		70	16	4·4
		50	12(7)	4·2
NC4	$1{\cdot}25\times10^5$	230	32	7·2
	(32)	215	40	5·4
		138	24	5·8
		136	32	4·3
		130	28	4·6
		100	12	8·3
NC4	$1{\cdot}5\times10^5$	180	32(4)	5·6
	(29)	115	24(3)	4·8
		115	20(3)	5·8
		108	24(3)	4·5
		108	20(2)	5·4
		108	16(2)	6·8
		186	34(2)	5·5
		100	17	5·9
		93	17(2)	5·5
		86	17	5·1
AX2	2×10^5	180	47(5)	3·8
	(25)	160	46(2)	3·5
NC4	4×10^5	255	64(8)	4·0
	(18)	210	68(8)	3·1
		158	32(8)	4·9
		115	32(8)	3·6
		65	20(8)	3·3
		74	12(8)	6·2
NC4	5×10^5	104	32	3·3
	(16)	104	28	3·7

* The intercellular distances, in μm, are given in parentheses.
† Where more than one determination has been made the number of determinations is indicated in parentheses.
‡ Since we show below that the signal is actually propagated in steps of about 57 μm this method of determining the velocity is subject to error, especially for pairs of cells that are close together.

TABLE 3

direct influence may not exceed 350 µm (Bonner, 1947 in Shaffer, 1962). The range of each relayed signal had been suggested by Cohen & Robertson (1971a), from their theoretical model and Gerisch's value of signal velocity (Gerisch, 1965) as being 10 µm. Measurements of this range and of the relay time were made in populations of different cell densities. Cells of D.discoideum NC4 were suspended in KK_2 buffer and spread in a thin film on NN-agar plates for development at 22° under illumination. Time-lapse films (1 frame/4 sec) were started at late interphase and continued throughout the aggregation stage; the agar plates were enclosed in humidity chambers specially fitted in the microscope. The stage of onset of aggregation was studied in repeated runs of the films during which individual cell responses were timed in a frame-by-frame analysis (4 sec intervals). Fig. 4 shows the results of two experiments in which cell density was varied 3 fold from 2.3×10^5/cm2 to 7.3×10^5/cm2. The amoebae, in both cases, responded in blocks of approximately 57 µm along the radii of the aggregation territory. The delay in movement responses (reflecting the relay time) from cell block to cell block was of 2, or more frequently 3, film frames. The time necessary for diffusion of the signal is known to be negligible since cAMP has a high rate of diffusion (4×10^{-6} cm2/sec, Cohen & Robertson, 1971a). The relay time is therefore less than but close to 12 sec; at both cell densities studied the range of influence of the relayed signal was 57 µm; the velocity of signal propagation that can be derived from these values (57 µm/12 sec = 4.75 µm/sec) is in agreement with the values derived from independent measurements presented above. Although the

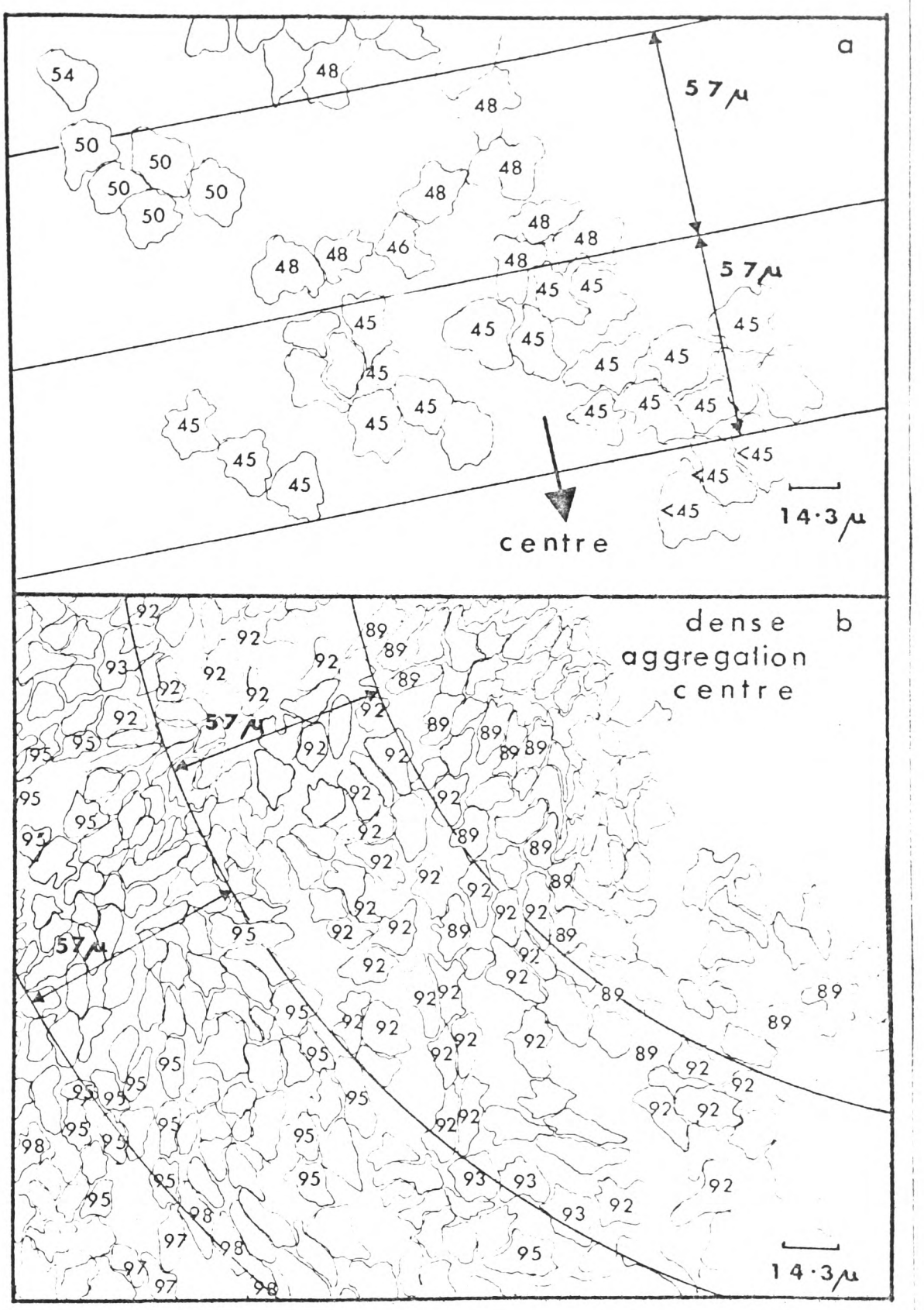

FIGURE 4

The range of the relayed signal and the relay time. A field of amoebae was traced from a time-lapse film of aggregating D.discoideum NC4. The film was run repeatedly through the same sequence and the frame (frames at 4 sec intervals) in which each amoebae started to move in response to a particular signal front was recorded; (a) and (b) correspond to independent experiments. In (a) unnumbered amoebae showed no marked movement response. In (b), not all of the amoebae were analysed and in many cases discrete movement steps could not be distinguished owing to the close proximity of the cells. Cell densities: (a) 2.3 x 10^5 amoebae/cm2; (b) 7.3 x 10^5 amoebae/cm2. The lines have been drawn knowing the position of the center and in such a way as to include as many 'simultaneously' responding amoebae as possible.

velocity of signal propagation may vary with cell density, the variation was only appreciable when extreme values of cell density were used. Within the range of densities selected for the analysis of signal relay, no variation in signal range or relay time could be noticed; below 2.3 x 10^5 cells/cm2 the population becomes too sparse for meaningful measurements of these parameters by this method.

1-3. Velocity of signal propagation at different cell densities. The measurements listed in Tables 2 and 3 have already shown the velocity of signal propagation increasing in populations of increased intercellular distance. In Table 4, more extensive data is presented; the mean values of velocity and the respective standard errors (see appendix 2) were estimated from measurements of movement-bands in several experiments with D.discoideum NC4 developing on NN-agar and from a mean movement duration of 100 sec. The degree of association between the values of signal velocity and the corresponding intercellular distances was assessed from the significance (student's t test) of the correlation coefficient estimated from 215 observations (appendix 3). Similar calculations

Table 4

Variation of velocity of signal propagation with intercellular distance

Cell density (amoebae/cm²)	Intercellular distance (μm)	Velocity* (μm/s)
5×10^4†	51	6·89 ± 0·67(3) 6·30 ± 0·42(7) 5·64 ± 0·41(5) 5·47 ± 0·39(16)
$1{\cdot}25 \times 10^5$	32	5·77 ± 0·18(43) 5·23 ± 0·20(7)
$1{\cdot}5 \times 10^5$	29	6·44 ± 0·40(7) 4·75 ± 0·10(10) 4·70‡(4)
$1{\cdot}9 \times 10^5$	26	5·28 ± 0·27(11)
$2{\cdot}5 \times 10^5$	22·5	6·48 ± 0·47(8) 5·63 ± 0·16(68) 4·76 ± 0·14(8)
5×10^5	16	5·51 ± 0·28(8) 4·70‡(5) 4·70‡(5)

* Measured movement band widths divided by 100 s, the movement duration (see above). The results are expressed as mean velocities with standard errors for 95% confidence limits. Separate experiments are recorded on different lines. Number of determinations are in parentheses.

† The wave pattern in fields of this density is not visible to the naked eye but can be seen clearly under the microscope. Aggregation is delayed, compared with fields of higher densities, and can take up to 13 h.

‡ No variation was observed.

were applied to the values of velocity and intercellular distances shown in Tables 2 and 3 (respectively appendixes 4 and 5). The two parameters appeared to be positively correlated ($0.02 > P > 0.01$). However, the increments in velocity did not reflect directly the increments in intercellular distance as can easily be noticed in Tables 2, 3 and 4.

1-4. The refractory period for movement response; the signal is pulsatile. Shaffer (1962) was the first to suggest in an analysis of the unisensal propagation of the signal, that cells of D.discoideum should become refractory some time after being stimulated. Robertson et al. (1972a) and Durston (1974a) presented evidence for a refractory period in signal relay and showed that it decreased during aggregation from about 6 to 2 minutes; this type of refractoriness ensured the outward propagation of the signal. It was shown (Alcantara and Monk, 1974; this thesis) that signal relay occurs shortly (12 sec) after initiation of the movement response. The maintenance of a centripetal movement for 100 sec seems to require that cells become refractory to changes in direction for not less than 12 sec from the time they emit their own signal. Attempts were made to detect and measure this period of refractoriness.

Ax_2 cells suspended in KK_2 buffer were placed on NN-agar plates in volumes of 10 µl and incubated. At the aggregation stage, when the cells were showing a 3 minute-pulsatile movement, each population was challenged with an artificial source of cAMP (2 µl of a 10^{-3} M cAMP solution) situated approximately 1mm from its periphery. The aggregation field was filmed (1 frame/ 4 sec) under 125 x magnification for 4 hours starting from 30 minutes before the time when cells were challenged with cAMP. The film

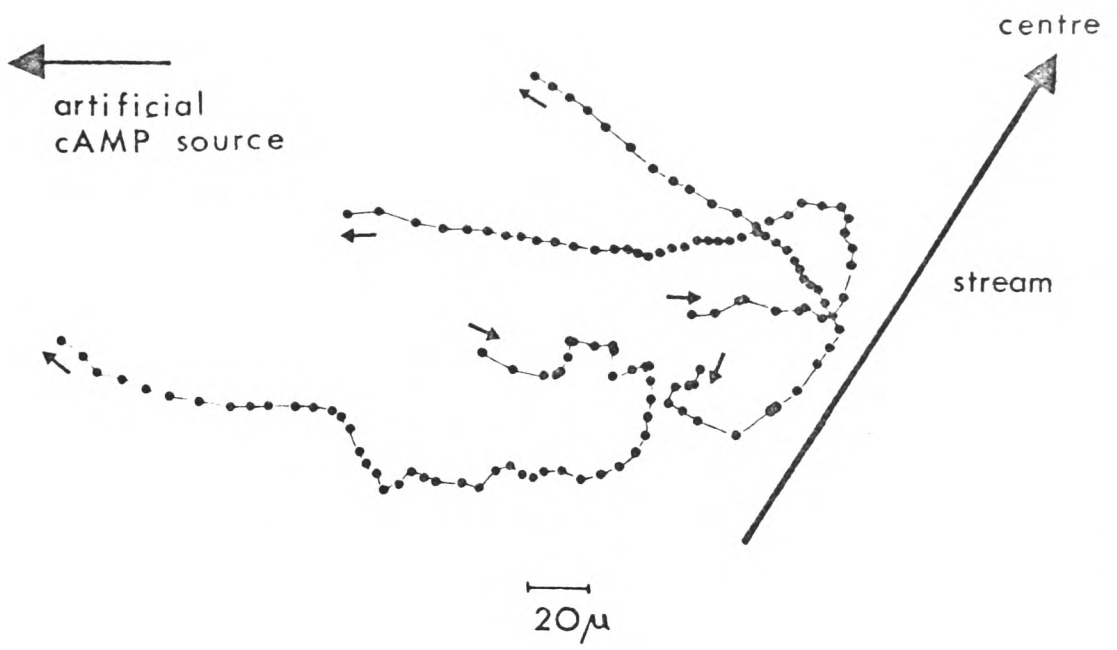

FIGURE 5

Analysis of a time-lapse film showing the path-tracks of three Ax_2 amoebae initially carrying out discrete movement steps towards a natural centre with a period of about 3 min. A small filter-paper square saturated with a cAMP solution (2 μl of a 10^{-3}M solution) was placed in the field at a distance of 1 mm in the direction indicated. Within 25 min the amoebae were diverted from the natural centre by the artificial cAMP source and moved continuously and with increasing speed towards it. Later, outside the field shown, their speed reached a maximum of 0.5 μm/sec. The points represent the position of the centre of each cell at 60 sec intervals.

was analysed at 60 sec intervals (15 frames) and the paths corresponding to the successive positions of the cell centres were recorded. Fig. 5 shows three of these path tracks in one of the several experiments carried out. This analysis shows (1) a change from pulsatile to continuous movement in response to the artificial cAMP source; (2) a turning-round movement towards the artificial source of attractant and (3) a progressive increase in cell velocity; at a later stage, the cell speed reached the maximum value of 0.5 µm/sec. This analysis not only provided further evidence for the pulsatile nature of the natural signal but also demonstrated that the refractory period for movement, to exist must be less or equal to the duration of the movement response (100 sec). The turning-round movement might be due to the geometry of the field; no consistent indication of cell polarity in terms of a fixed front region could be observed. The interpretation of the variation in cell speed with time of response in artificial gradients of cAMP is currently being investigated.

A different sort of approach made it possible to decrease this upper limit for the refractory period of movement. The tracks of cells frequently changing direction were followed by time-lapse film analysis; the cells were either responding to signals emanating from one neighbour stream in conditions of spontaneous aggregation (Table 5, cells 1-5) or they were responding, alternately and in zig-zag, to two adjacent streams moving continuously toward a continuous source of cAMP (Table 5, cells 6-8). In the first case the cells moved obliquely to the stream in direction of the centre, changed to a nearly perpendicular path

Consecutive directional path times in cells stimulated from different directions

Strain	Cell	Directional path time* (s)	Minimum directional path time (s)
NC4	1	24,12,20,12,16,16,20,16	12
	2	16,16,12	12
	3	16,12,36,16,24, 32, 24,20	12
AX2	4	12,20,32	12
	5	16,20,16,12,32,44,44,28, 40,28	12
	6	68,16,36,28,24,60,20,32,20, 28,24,20,28,32,44,40,24	16
	7	12,36,36,24,16,12,56,44,56, 24,32,16,120,16,20,16	12
	8	16,48,48,48	16

* The short stationary periods between directional displacements which occur in some directional changes were added to the path time in the new direction.

Table 5

and then moved away from the centre along the stream as they were stimulated by the propagating signal from the stream being relayed outward. These cells frequently abandoned the stream they had temporarily approached to rejoin it and form stable contacts at the following signals. The minimum time for unidirectional tracks was most commonly 12 sec. (Table 5, Alcantara & Monk, 1974). Therefore the refractory period for movement is probably not more than 12 sec. However, it was observed that this is also the minimum time required for cell displacement, that is, for contraction of the rear of the cell after pseudopod formation; the 12 sec. value is therefore the limit of the method and the refractory period for changes in direction may possibly be smaller.

Conclusions

The patterms developed during aggregation in Dictyostelium directly reflect some aspects of the physiological mechanism involved in intercellular communication e.g. the frequency of signalling, the ability to relay signals and the ability to form stable cellular

contacts. A quantification of some of the parameters involved in the process of signalling during aggregation was attempted in the hope it might help in clarifying or revealing cell properties and population-dependent mechanisms of control. It was observed that the range of the signal was not controlled in such a way that the signal had to be strictly transmitted from cell-to-cell; this has been proved by direct measurement of signal range and it also follows from the fact that the increments in signal velocity are not proportional to the increments in intercellular distance (Tables 2, 3 and 4). However, the value of the signal velocity was shown to be somehow dependent on the density of the population. This variation may be explained in at least three ways:

(1) The amplitude of the signal (cAMP out-put) varies according to the cell density and its range of influence is controlled enzymatically (membrane-bound and extracellular PDE); the balance between these two factors would favour increased signal ranges at low cell densities.

(2) The amplitude of the signal is constant (feed-back control of cAMP synthesis or secretion) and decreased activities of signal controlling enzymes allow for enlarged signal ranges at low cell densities.

(3) The amplitude of the signal increases during interphase (signal range may or may not be controlled enzymatically); sparser (later aggregating - see below) populations might, consequently, present an increased signal velocity due to the increase in signal range. Some evidence for a feed-back control on the amplitude of the signal resulted from Shaffer's observations (1962) that the concentration of attractant (estimated from the

capacity to attract responding cell populations) did not depend on cell mass. Though it has been widely admitted that extracellular PDE may be involved in the formation of cAMP gradients (Bonner et al., 1969; Gerisch et al., 1972; Chassy, 1972) it has not been proved that this enzyme may affect signal range. Nanjundiah (personal communication) has calculated that the ratio between the rates of cAMP diffusion and cAMP hydrolysis by extracellular PDE is of the order of 10^{-3} which renders any control on signal range of this enzyme impossible. Membrane-bound PDE seems to control the time for hydrolysis of cAMP in between pulses rather more effectively than the extracellular form of the enzyme (Malchow, Fuchila & Nanjundiah, 1975) but, again, it appears to have no role in the control of signal range. On the basis of this evidence, hypothesis (3), in the modality of no enzymatic control of signal velocity, might be favoured.

It was shown that signal relay occurred shortly (12 sec) after the movement response to the stimulatory signal was initiated and long (approximately 88 sec) before it ceased. This value supports our observation that signalling does not cause the appearance of vacant zones in aggregation fields; cells moving at their highest speed (0.5 μm/s) would move, in 12 sec, a distance of 6 μm, which is not enough to create a visible pattern even in Gerisch's conditions where the hypothetical vacant zones are shown as 192 μm-wide bands. The relay time in conjunction with the measurements of relay zones permit to calculate a value for velocity of signal propagation (4.75 μm/sec) which agrees with independent values also presented in this thesis.

The cells respond with oriented movement to a single signal front for a period of approximately 100 sec. Meanwhile they amplify the signal and by so doing the signal is propagated outward. The uni-sensal propagation of the signal makes necessary the existence of a refractory period for signal amplification (Shaffer, 1962; Robertson et al, 1972 a; Durston, 1974 a). It was demonstrated (Alcantara & Monk, 1974; this thesis) that cells are not refractory to changes in direction for more than 12 sec. Gerisch and co-workers (1975 c) also showed that cells may form distinct and differently oriented pseudopods in as little as 5 sec. In conjunction these results seem to indicate that cells are never refractory to new orientations. If cells are not refractory to reorientation they should stop when emitting a signal and they should reverse direction when the adjacent-outer relay zone signals, but they do not. Tentatively two hypotheses may be suggested to account for the 100 sec period of continuous elongation and orientation:

(1) the signal is not instantaneous, the cells attaining a peak in the release of attractant some time after initiation of secretion; this extended period of signalling and the relay time of 12 sec. ensure a period of oriented movement of 100 sec;

(2) the signal is emitted instantaneously and the process of emission makes the cell refractory for changes in direction for 12 sec (minimum time compatible with uni-sensal orientation in movement); in the conditions reported by Gerisch et al (1975 c) probably no signal relay was occurring, as cAMP concentration was maintained high by the conditions of the experiment (for evidence of high concentrations of extracellular cAMP inhibiting cAMP

secretion see Gerisch & Hess, 1974b). This phenomenon of maintained cell orientation over periods of 100 sec is open to further investigation.

The value of signal velocity varied, depending on cell density, from 3.1 to 8.9 µm/sec (186-534 µm/min). Only at low, critical, densities did these values agree with the value (500 µm/min) calculated by Shaffer (1962) and they were always substantially different from the value (43 µm/min) estimated by Gerisch (1965). It remains to be clarified whether these values reflect strain differences or different experimental conditions.

2. Extracellular cAMP-phosphodiesterase and the acceleration of differentiation towards aggregation competence in Dictyostelium discoideum

2-1. AF. Activity. The period of differentiation which takes place for several hours after the initiation of starvation is commonly described as the pre-aggregation stage or interphase. It has been reported (Konijn, 1968; Alcantara & Monk, 1974) that the duration of this period depends on cell density but it was not always possible to distinguish between effects due to variation in the size of the population and to variation in cell density. An extended pre-aggregation stage at low densities might be due to factors limiting aggregation per se (e.g. appearance of autonomous centres) or it could be the result of a slower rate of differentiation toward the stage of aggregation competence.

2-1-1. Effect of cell density on the duration of the pre-aggregation stage (KK2 treated cells). Three experiments were conducted in which the effects of varying cell number and cell density on the

duration of interphase (t_{agg}) could be judged separately. Ax_2 cells were harvested from exponentially growing cultures, washed and resuspended in KK2 buffer at densities differing successively by a factor of two. Ten and five microlitre aliquots were taken from each suspension (5 replicas of each) and deposited on NN-agar plates; incubation was at 22^{o} in the light. The appearance of the first signs of aggregation - the formation of 3-4 cell streams - was monitored by microscopic observation. In Fig. 6 (broken lines) t_{agg} is the average time of onset of aggregation in each 5 replicas; the deviation from this value did not exceed 1/4 hour.

The effect of cell density alone is made evident by comparing t_{agg} in populations of the same number of cells but with a two fold difference in density (5 µl population - close symbols - compared to a 10 µl population - open symbols - at a density immediately below). When cell density ranged from 10^9 to 1.25×10^6 cells/ml, the duration of interphase varied from 4½ to 14 hours. It seems clear that at densities greater than 2.5×10^6/ml, cell density, and not cell number, was the major factor determining the length of interphase. At densities of approximately 10^7 cells/ml or more t_{agg} appeared to be invariant with respect to further increase in cell density. In populations of 2.5×10^4 cells or less, both cell density and cell number appeared to be affecting t_{agg}.

Having distinguished the effect of cell density on t_{agg} it was asked if this effect was related to the process of aggregation itself or to the rate of differentiation. A direct effect on aggregation could arise, for example, if the range of the signals inducing aggregation increased progressively during interphase;

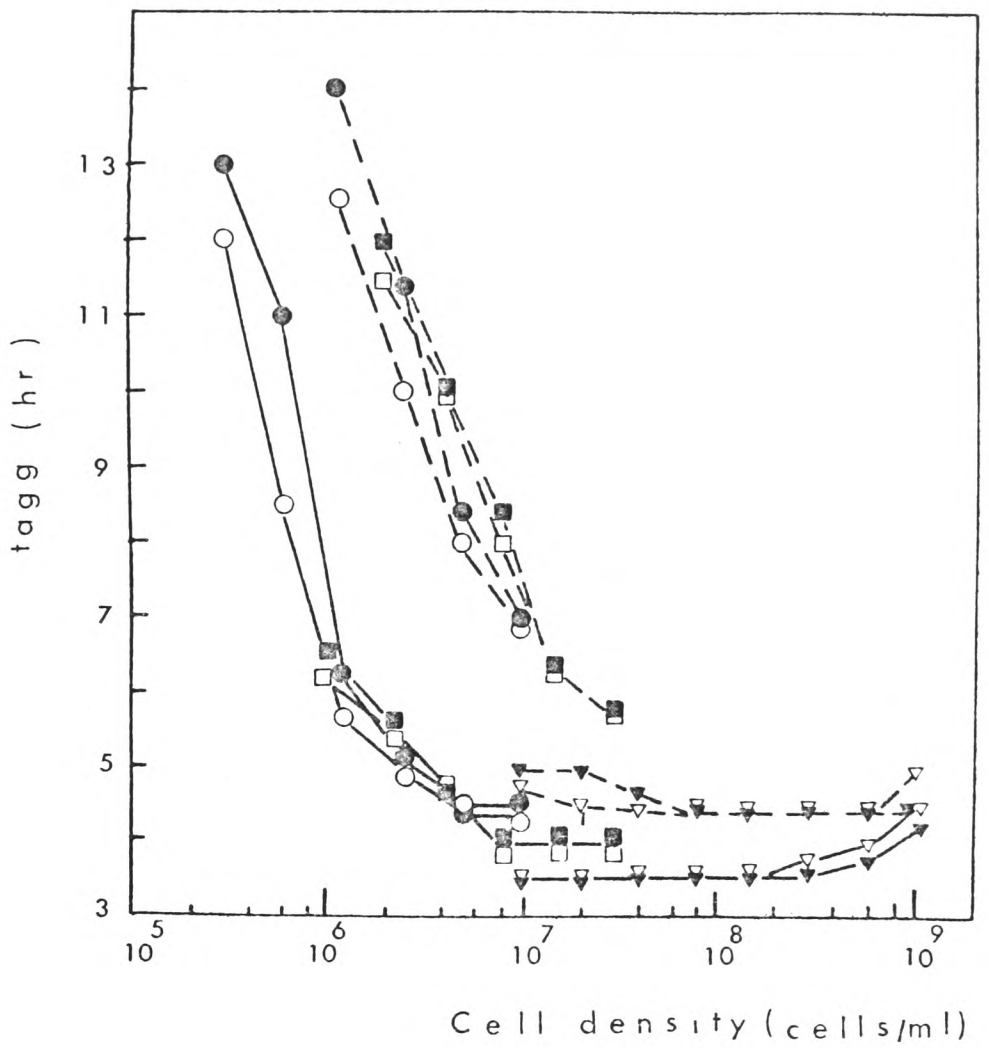

FIGURE 6

Dependence of duration of interphase (t_{agg}) on cell density. Development was initiated in Ax_2 cells suspended either in KK2 buffer (----) or AF solution (——). Three experiments (o,●; □ ,■; ∇ ,▼)were conducted. After adjustment to a desired initial cell density both cell suspensions were successively diluted in the appropriate medium. From the series of cell suspensions samples of 10 µl (open symbols) and 5 µl (closed symbols) were placed on NN-agar plates and incubated at 22^{o} with illumination. Time of onset of aggregation (t_{agg}) was monitored by microscopic observation. Points represent the mean t_{agg} for each 5 replicas; deviation from the mean did not exceed ¼ hour.

aggregation would then start earlier in populations with shorter intercellular distances. Alternatively, t_{agg} might be related to the rate of differentiation during interphase; this could depend on accumulation of extracellular factors in the medium or on the frequency of cell contacts during random movement.

2-1-2. Duration of the pre-aggregation stage in A F. treated cells. To test whether the medium contained an extracellular factor which controlled t_{agg} Ax_2 cells were harvested, washed and suspended in KK2 buffer at approximately 10^7 cells/ml. The cells were kept aerated and dispersed by agitation in a rotary shaker at 160 rev/min and were incubated at 22°. At the stage of aggregation-competence, shown by prompt aggregation after deposition of small aliquots on NN-agar, the medium was collected free of cells by centrifugation, first at 260 g for 1 min and then at 10,000 g for 20 min at 4°. When used as the suspending solution for freshly harvested cells, this medium reduced the duration of interphase and accelerated development of aggregation competence. It was therefore called AF solution (AF for acceleration factor or factors).

The effect of AF on duration of interphase in populations at different cell densities is shown in Fig. 6 (full lines). Ax_2 cells were suspended at t_o in AF solution at densities corresponding to the controls for which the suspending medium was KK2 buffer (broken lines); 10 µl and 5 µl aliquots were placed on NN-agar plates and incubated. The experiments with AF suspended cells and KK2 buffer suspended cells were run in parallel, that is, cells were harvested from the same culture and densities were adjusted to

the same value.

In all cases t_{agg} was markedly decreased when cells were suspended in AF solutions; the difference in t_{agg} in relation to the KK2 control increased progressively with the decrease in cell density from 1 hour to 8 hours. In AF populations at approximately 10^7 cells/ml t_{agg} was minimal and so was the difference in t_{agg} in relation to the KK2 control (1 hour). These results were consistent with AF being a cell-produced factor. The small increase in t_{agg} in AF populations at densities above 3×10^8 cells/ml may be attributed to difficulty in distinguishing the initial aggregation stage of 3-4 cells streams in cell multilayers (confluence at 3×10^8 cells/ml.).

Treatment with AF extended the critical density for aggregation to a density of 1.25×10^4 cells/cm2 (3×10^5 cells/ml) corresponding to an intercellular distance of 101 µm. Lower densities were not tested.

The cell density proved to be an important factor controlling the duration of interphase even when cells were suspended in AF solution. A density of $5\text{-}7 \times 10^6$ cells/ml was selected for subsequent AF assays; at this density small variations in the volume of the population had no effect in the value of t_{agg} (Fig. 6). Ax_2 cells in the exponential phase of growth, harvested according to the procedure described in "Materials and Methods" and suspended at the same density could develop aggregation competence at different times on different occasions. This cell-inherent variation occurred both in KK2 and AF treated cells, but the effect of AF was always marked.

Conclusions

It was shown that the duration of the pre-aggretation stage (t_{agg}) may vary from 4½ to 14 hours (or more) when cells are suspended in KK2 buffer. Cell density (cells/unit of volume) was a major factor in this variation; the total number of cells in a population only affected t_{agg} when the population was constituted by 2.5×10^4 cells or less. Konijn & Raper (1961) have reported the dependence of aggregation on cell number and density (cells/unit area). The results in this thesis are not to be compared with those of Konijn & Raper for two reasons (a) values of cell density here correspond to number of cells per unit volume of medium. In Konijn & Raper's work, the medium was absorbed by agar; density, in such conditions, corresponded to number of cells per unit area; (b) I have studied the duration of interphase and not the capacity for aggregation regardless of the time taken to start it. However, the general conclusion from their work seems to be that the two controlling factors, cell number and cell density, can counter-balance each other in unfavourable conditions; this is supported by data presented here.

The extracellular medium of aggregation-competent cells contains an accelaration factor(s) (AF) which shortens the duration of interphase (see below the time course of changes in AF activity during interphase). Cells suspended in this medium at densities ranging from 3×10^5/ml to 10^9/ml aggregated one to eight hours earlier (depending on cell density) than the respective controls (cells suspended in KK2 buffer). Even when the cells were treated with AF solutions, t_{agg} increased with decreasing cell density. This observation may admit several explanations: (a) the time needed

for formation of streams after initiation of aggregation increases with intercellular distance (this varied from 101 µm at 3×10^5 cells/ml down to a state of confluence at 3×10^8 cells/ml or more). This reason alone could hardly account for the 9 hours of variation between the two extremes of duration of interphase; (b) the range of the autonomous or relayed signals may increase (up to a limit) during interphase and this may influence t_{agg} independently of AF; (c) fixed proportion or fixed rate of appearance of autonomous centers in a population may delay aggregation in sparse preparations; (d) AF is active only for a limited period of time (due to inactivation or diffusion) after which the inability of the population to cope with the prevailing conditions would result in delayed onset of aggregation. The results in this thesis (see below) suggest that the cAMP oscillator is not under developmental control; this argues against hypothesis (c). The other hypotheses are open to investigation. Another point of interest is the reason for the minimal one hour difference in the duration of interphase between AF and KK2 treated cells even when cell density is progressively increased beyond the stage of confluence. This period may correspond to the minimal time required for synthesis or induction of AF.

2-2. Characterisation of AF

2-2-1. Preliminary characterisation of AF. Ten minutes of incubation of AF solution at 80° totally destroyed AF activity; in fact the duration of interphase in cells suspended in the pre-heated medium might be increased by one or two hours in relation to the KK2 buffer control. This treatment also inactivates PDE and leads to accumulation of free extracellular PDE-inhibitor (Riedel et al. 1972).

Addition of ammonium sulphate to AF solutions to a level of 60% saturation (390 g of solid ammonium sulphate per 1 litre of solution) precipitated AF and PDE activities.

Gel filtration of a concentrated t_{10} supernatant on a Sephadex G 25 column (55 cm x 1.5 cm) with a fractionation range of 1,000-5,000 daltons, showed that AF activity was eluted in the void volume (33 ml). None of the other fractions (1.3 ml) showed any AF activity nor could they increase the AF activity in the active fractions.

Ultrafiltration of AF solutions through membranes with a nominal retentivity of 1,000 daltons (Millipore PSCA membranes) retained AF and PDE activities. Amicon PM 10 membranes with a nominal retentivity of 10,000 daltons proved to be equally effective in concentrating both activities.

A non-specific protein was tested to see if it could mimic the effect of AF solution on the duration of interphase. A bovine serum albumin solution with approximately the same protein content as the AF solutions (0.035 mg protein/ml) was tested in AF assays. No stimulatory or inhibitory effects on the duration of interphase were observed; t_{agg} for the bovine serum albumin-treated cells was similar to t_{agg} in the KK2 buffer control.

2-2-2. Fractionation of AF solutions

2-2-2-1. DEAE cellulose chromatography. A concentrated t_{10} supernatant was purified on an anion-exchange column (Whatman DE 52). Fig. 7 shows the results of AF and PDE assays on the 50 4 ml-fractions eluted with a gradient of 0.0 - 0.4 M KCl in 10 mM triethanolamine-HCl buffer pH 6.8. The absorbance for each fraction at 280 nm is also shown. PDE activity formed two peaks but only the last eluting

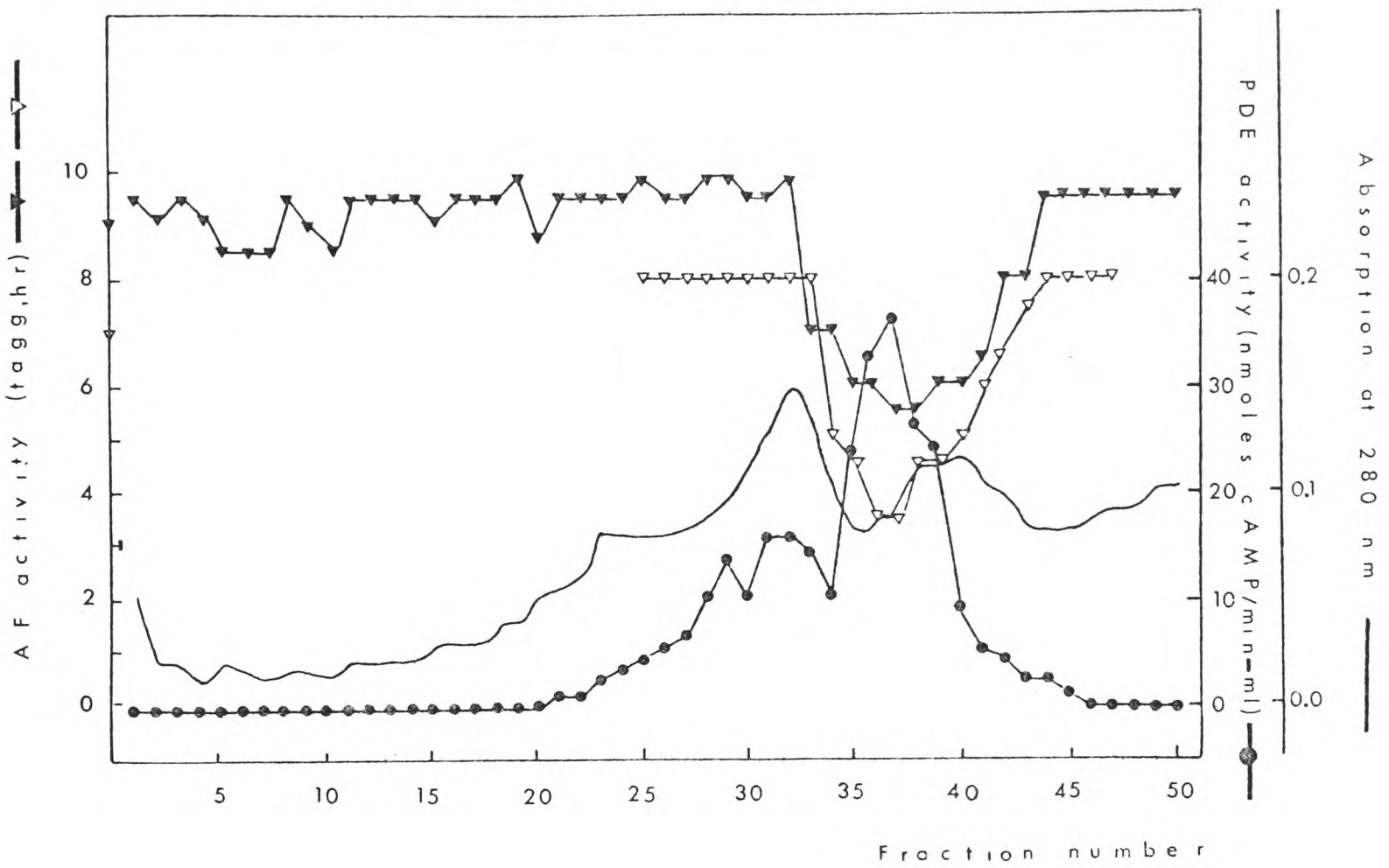

FIGURE 7

DEAE cellulose chromatography of a t_{10} supernatant. The column was eluted with a continuous gradient of 0.0 - 0.4 M KCl in 10 mM triethanolamine-HCl pH 6.8. AF activity was measured in two experiments (▽ , ▼); t_{agg} for the KK2 controls is shown on the ordinate; (●)PDE activity; (——) absorption at 280 nm. Deviation from the mean t_{agg} was not greater than ¼ hour.

one coincided with AF activity. Dr. G. Bazill determined the Km values for these enzymes and found that only the enzyme in fractions 36-39 had the low Km value (approximately 4 x 10^{-6}M cAMP) reported by other authors (Riedel et al. 1972). The enzyme in fractions 28-35 had an apparent Km of about 10^{-4}M cAMP.. AF activity clearly peaked between fractions 34 and 43, coinciding exactly with the peak of the low Km form of PDE. The specific activity of extracellular PDE in fraction 37 (364 units/mg protein) was 10 fold increased in relation to the initial supernatant.

2-2-2-2. Filtration on Sephadex G 200. Further purification of AF, on the basis of molecular size alone, was attempted. After chromatography on DEAE cellulose as above, 0.5 ml aliquots of fractions 35-39 were pooled and applied to a Sephadex G 200 column (55 cm x 1.5 cm; fractionation range of 5,000 to 800,000 daltons). Elution was carried out with 0.017 M sodium/potassium phosphate buffer pH 6.1. Figure 8 shows the profiles of PDE activity and AF activity (points correspond to the mean t_{agg} in 3 replicas; deviation from the mean $t_{agg} \leqslant \frac{1}{4}$ hour) and the absorbance at 280 nm (measured in a Beckman DB G spectrophotometer). Peaks of AF and PDE activities were again coincident, now appearing between fractions 18 and 30. Blue dextran 2000 peaked in fraction 18 (void volume 22 ml) and NaN_3, the end marker, was eluted in fraction 60 (72 ml). PDE in fraction 23 had a specific activity of 776 units/mg protein which represents a two fold purification in relation to fraction 37 after DEAE cellulose chromatography.

No dithiothreitol (DTT) was added before or after fractionation. Although 0.2 mM DTT appears to prevent conversion of the low Km into the high Km form of extracellular PDE (Chassy, 1972) it was avoided

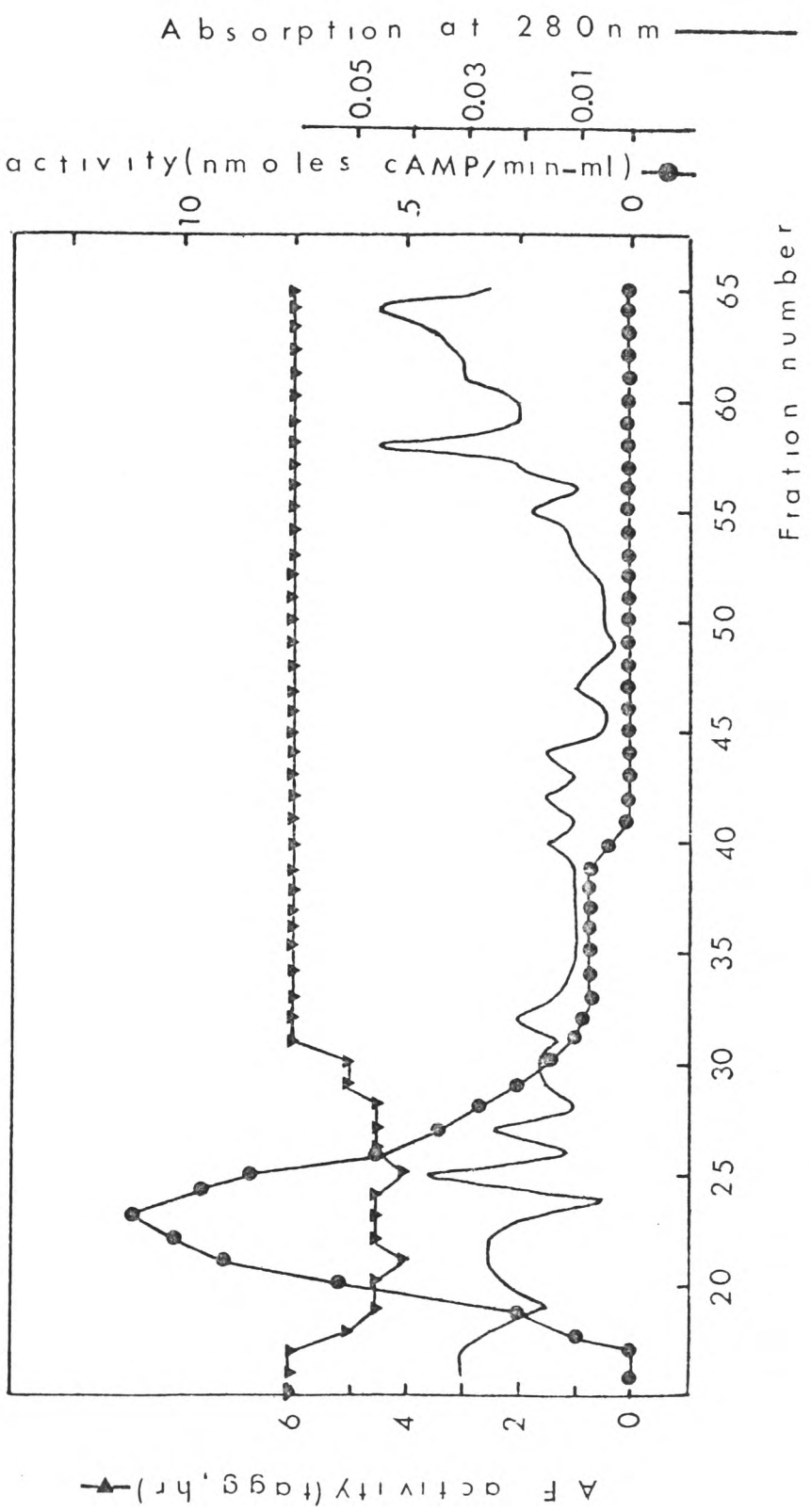

FIGURE 8

AF and extracellular PDE purification on a Sephadex G 200 column (55 cm x 1.5 cm) after DEAE cellulose chromatography. 1.2 ml fractions were collected during elution with 0.017 M Na/K phosphate buffer pH 6.1. Blue-dextran 2000 eluted in fraction 18 (void volume, 22 ml) and the end marker (NaN_3) appeared in fraction 60 (72 ml). (▼) AF activity expressed as mean t_{agg} for 3 replicas; deviation from the mean $t_{agg} \leq \frac{1}{4}$hr. Duration of interphase for the KK2 control is indicated in the ordinate. (●) PDE activity; (——) absorption at 280 nm.

because DTT interferes with the process of aggregation in the AF assay. According to the same author the extent of conversion of low Km to high Km forms of the enzyme at 4^{o} in three days (the time taken for DEAE cellulose and Sephadex G 200 fractionations) is negligible.

2-2-2-3. Disc electrophoresis. A 93 x concentrated t_{10} supernatant was analysed by electrophoresis in 7% polyacrylamide gels, under conditions in which separation depended both on molecular charge and size. Fig. 9 shows the profiles of AF and PDE activities obtained. Each point corresponds to the activity in 1 mm slice eluted in 0.5 ml of 10 mM triethanolamine-HCl buffer pH 6.8 Each point in the AF curve corresponds to the mean t_{agg} in 3 replicas; the deviation from the mean was $\pm$ ½ hour.

Two peaks of PDE activity were obtained. Possibly these two peaks correspond to the low and high Km forms of PDE. Activity coincided with the first, less mobile peak of PDE. Stained replica gels showed 16 protein bands at distances from the origin ranging from 0.4 cm to 8.5 cm (bromophenol marker at 10.3 cm). No protein band could be distinguished in the stained gels at 1.4 cm from the origin (fraction 14). The second peak of PDE (fractions 15-20) might correspond to a dense protein band at 1.7 cm from origin.

2-2-3. AF activity of rat brain PDE. The fractionation of AF solutions provided evidence for the association of AF with the low Km form of PDE. Three hypotheses could be formulated: (1) AF is simply PDE activity, (2) AF depends on a second active site in the PDE molecule or (3) AF corresponds to a complex formed by PDE and

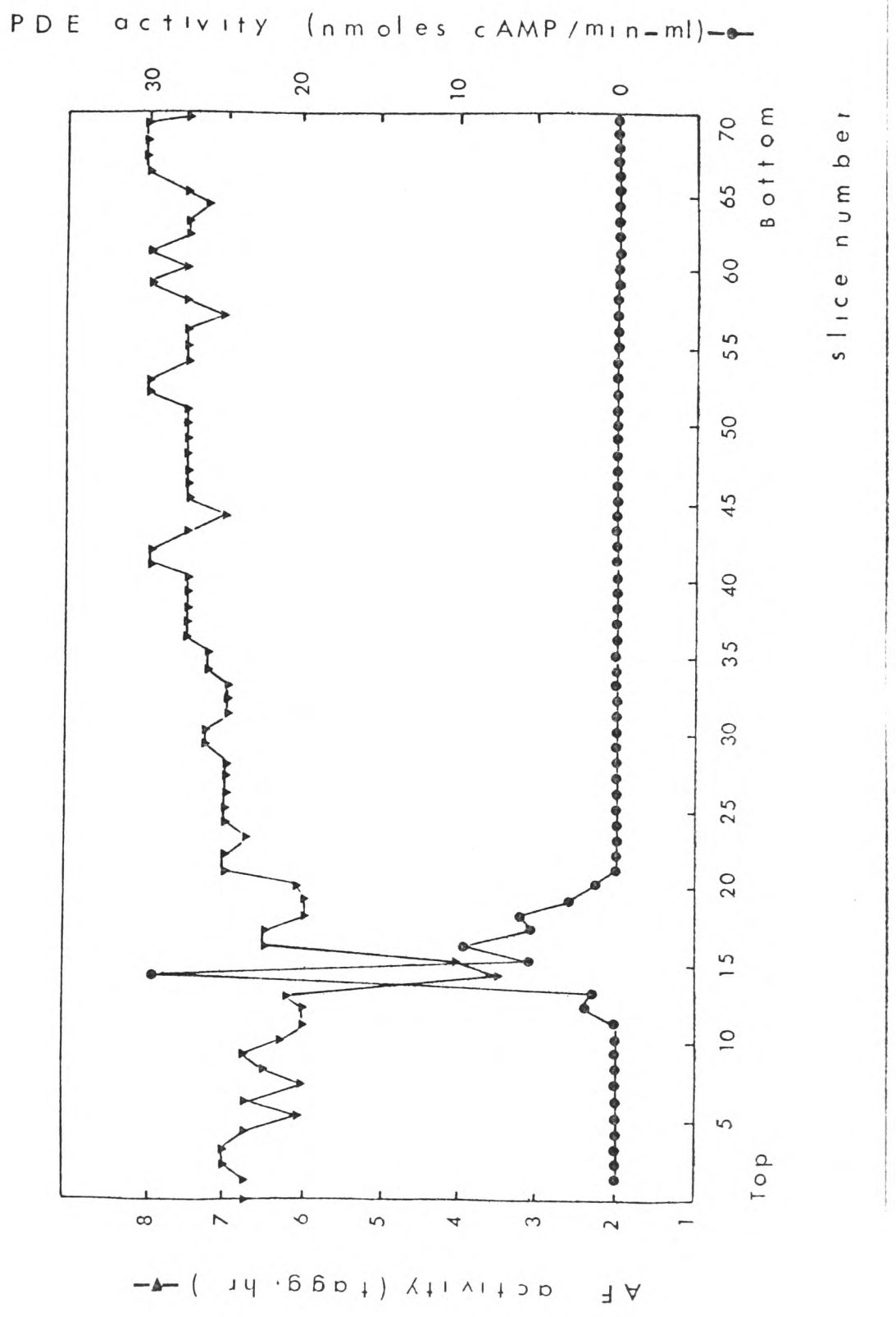

FIGURE 9

Disc electrophoresis of an AF solution (93 x concentrated t_{10} supernatant) on 7% polyacrylamide gel. (●) PDE activity; (▽) AF activity: points represent the mean time of onset of aggregation (t_{agg}) for three replicas (maximum deviation from the mean = ½ hr.). The point in the ordinate represents t_{agg} in the KK2 control.

other molecules. To distinguish between these possibilities PDE of a different origin but with a Km comparable to that of slime mould PDE was tested for AF activity. Rat brain PDE was prepared according to Thompson & Appleman (1971). The enzyme had a Km of, approximately, 10^{-6}M cAMP and the activity of the preparation was 50 units/ml (determinations by Dr. G. Bazill). AF assays for different dilutions of this enzyme and for the controls (Ax_2 AF solution, KK2 buffer and imidazole buffer) were carried out on imidazole-agar as rat brain PDE activity is enhanced by imidazole and requires Ca^{++} and Mg^{++} (Cheung, 1967; Lin et al. 1974). Table 6 shows the t_{agg} values corresponding to rat brain PDE solutions in four separate experiments in comparison to the three controls. PDE activity is indicated in brackets.

Rat brain PDE proved to be as effective as slime mould PDE in promoting acceleration of the aggregation-competence state.

2-2-4. Time course of changes in AF and extra-cellular-PDE activities during interphase. If AF and extracellular PDE activities were dependent upon different molecules, the kinetics of changes in the two activities during interphase might be expected to differ. The activity of extracellular PDE during interphase had been reported to follow a different time-course according to the conditions of incubation: shaken suspensions behaved differently to preparations on solid substrata (Malkinson & Ashworth, 1973a). For this reason Ax_2 cells suspended in KK2 buffer were incubated in both conditions. Cell suspensions at different densities (3×10^6/ml, 4×10^7/ml and 8×10^7/ml) were agitated at 160 rev/min at 22°. In other experiments, aliquots of cell suspensions at 4×10^7/ml and 8×10^7/ml were placed and

Table 6

rat brain PDE t_{agg}	Ax_2 AF sol. t_{agg}	KK2 buffer t_{agg}	Imidazole buffer t_{agg}
7(8)	7(12)	9½(o)	10(o)
5(50)-6(20)	5(18)	8 (o)	8(o)
6(4) -6(20)	5(9)	8 (o)	8(o)
6(4) -6(20)	6(9)	8 (o)	8(o)

AF activity of rat brain PDE. AF assays were conducted on imidazole-agar with Ax_2 cells suspended in rat brain PDE solutions or in control solutions (Ax_2 AF solution, KK2 buffer and imidazole buffer). PDE activity in each solution (units/ml) is indicated in brackets. t_{agg} (hr) is the mean aggregation time in five replicas; deviation from the mean did not exceed ¼ hour. Each line corresponds to a separate experiment.

spread on NN-agar plates (0.5 ml of cell suspension per plate); these plates were incubated at 22° in humidity chambers. Samples of the medium were collected at intervals of one hour according to the procedure for preparation of AF solutions. In the case of the NN-agar preparations the cells were detached from the agar surface by gently passing a glass rod over the liquid film before collection of the medium (separate plates were used at different times). These samples were assayed for PDE and AF activities. As a control in the AF assay cells were suspended in KK2 buffer. The duration of interphase in each different preparation was

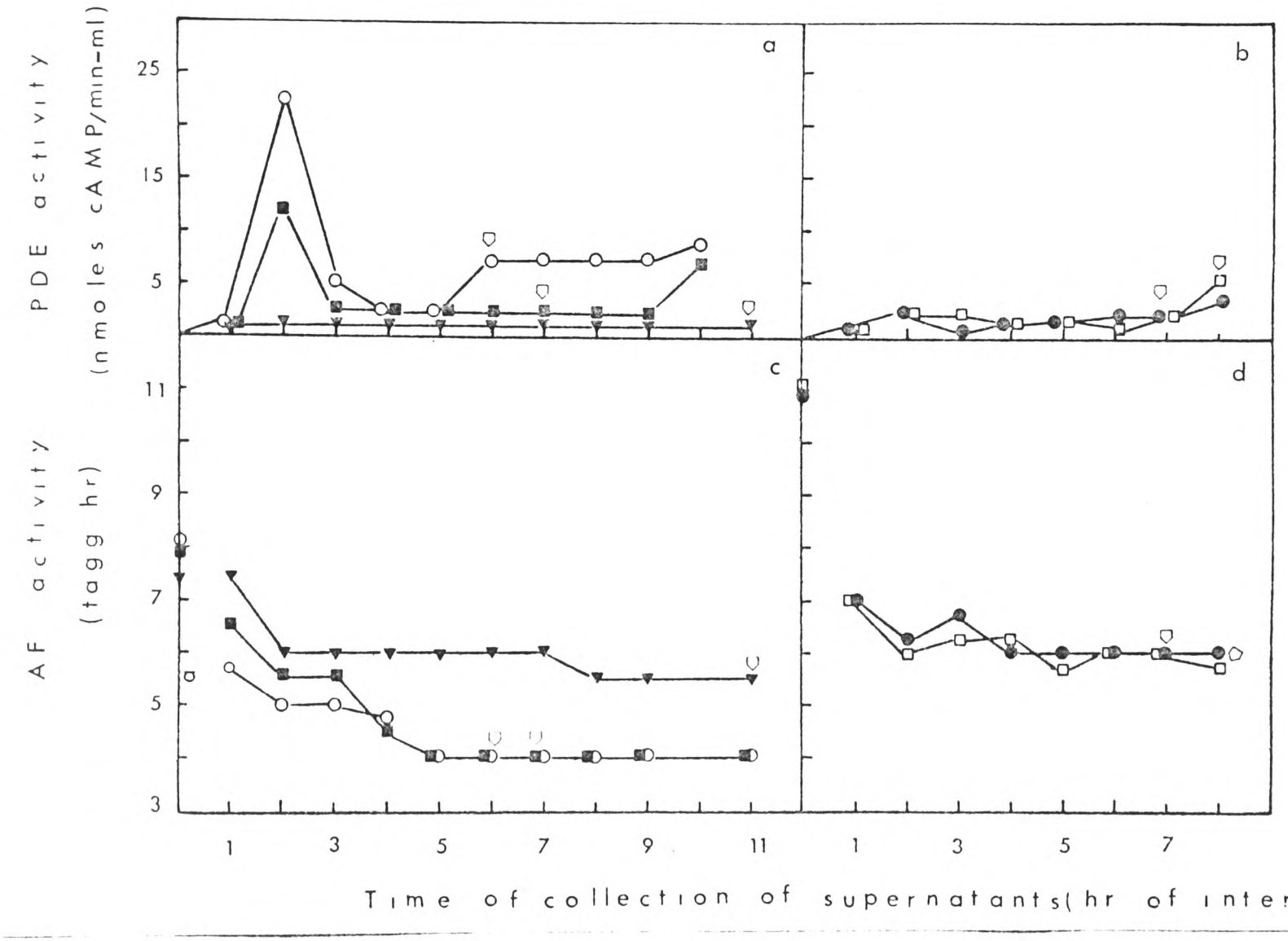

FIGURE 10

AF and PDE activities in the extracellular medium during interphase. Exponentially growing Ax_2 cells were resuspended in KK2 buffer at different densities: (o,●) 8×10^7 cells/ml, (□ , ■) 4×10^7 cells/ml, (▼) 3×10^6 cells/ml. The cells were either kept in suspension with agitation (a,c) or spread on NN-agar plates (b,d). At several times of interphase aliquots of the extracellular medium were collected and stored as AF solutions. These supernatants were later assayed for PDE activity (a,b) and for AF activity (c,d). Each point in (c,d) represents the mean t_{agg} for five replicas; variation from the mean was $\lesssim \frac{1}{4}$ hr. Arrows indicate t_{agg} in the cultures from which the supernatants were collected.

recorded as a mean t_{agg} for 5 replicas.

Fig. 10 shows the kinetics of changes in PDE and AF activities in both conditions. In suspensions at 3×10^6 cells/ml the level of extracellular-PDE activity did not exceed 1.5 units/ml) (Fig. 10 a) and no peak in this activity could be detected. AF activity in the series of samples corresponding to this culture was low and showed a slight increase at t_8 (Fig. 10 c). Suspensions at 4×10^7 cells/ml or 8×10^7 cells/ml formed a peak of extracellular-PDE activity at t_2 and then the activity was decreased to a level of 2.5 units/ml (Fig. 10 a) due to the secretion of a specific inhibitor of this enzyme (Riedel et al. 1972). In some cases, exemplified here by the 8×10^7 cells/ml suspension, PDE activity could rise to 7.5 units/ml at the time of aggregation competence. AF activity in both suspensions increased until t_2 (Fig. 10 c) and not only did it not drop after this stage but it continued to increase, in one case significantly, until t_5.

Preparations on agar (Fig. 10 b,d) did not show the early peaks in extracellular-PDE activity; a level of 2.5 units PDE/ml was reached at t_2 and the activity remained at this plateau till the onset of aggregation when it increased to as much as 5 units/ml. AF activity in the medium increased till t_2 and did not show significant variation afterwards.

2-2-5. AF activity versus PDE activity. The experiment of Fig. 10 indicated that 2.5 units PDE/ml was still consentaneous with high AF activity. It was attempted to define what was the minimal PDE activity required for full AF activity.

It was also tested if extracellular PDE inhibited by its natural inhibitor was active as AF (this was a form of testing the

hypothesis of AF activity depending on a second active site in the extracellular-PDE molecule). This possibility had been discarded on the basis of the results with rat brain PDE. However, some of the results expressed in Fig. 10 revived the interest in the question not only because AF activity was not decreased after t_2 but also because it could be incremented till t_5, three hours after the peak in PDE activity.

Early (t_2) supernatants of Ax_2 cells were known to possess high extracellular-PDE activity and little extracellular-PDE-inhibitor (Riedel et al. 1973). For this reason these supernatants were used in the preparation of series of dilutions of PDE in KK2 buffer as well as for preparation of partial inhibited PDE. The specific inhibitor of extracellular-PDE was prepared and used according to Riedel & Gerisch (1971) and Riedel et al. (1972). In PDE inhibition series the final dilution of the supernatant was kept constant.

Fig. 11 shows the duration of interphase (t_{agg}) corresponding to different degrees of dilution of the PDE solution in 6 experiments (a) and to different degrees of inhibition of PDE in 5 experiments (b). Crude inhibitor was used in all but one (□) experiment for which a preparation of inhibitor partially purified by DEAE cellulose chromatography was utilised. The AF assays were conducted in parallel in (a) and (b) using the same stock suspension of responding Ax_2 cells in all experiments represented by the same symbols. t_{agg} for the KK2 controls are shown on the ordinate.

An activity of 2.5 units/ml PDE proved to be the average minimal activity still compatible with maximum AF activity both after dilution or inhibition of PDE. Populations with a longer t_{agg}, as in the KK2

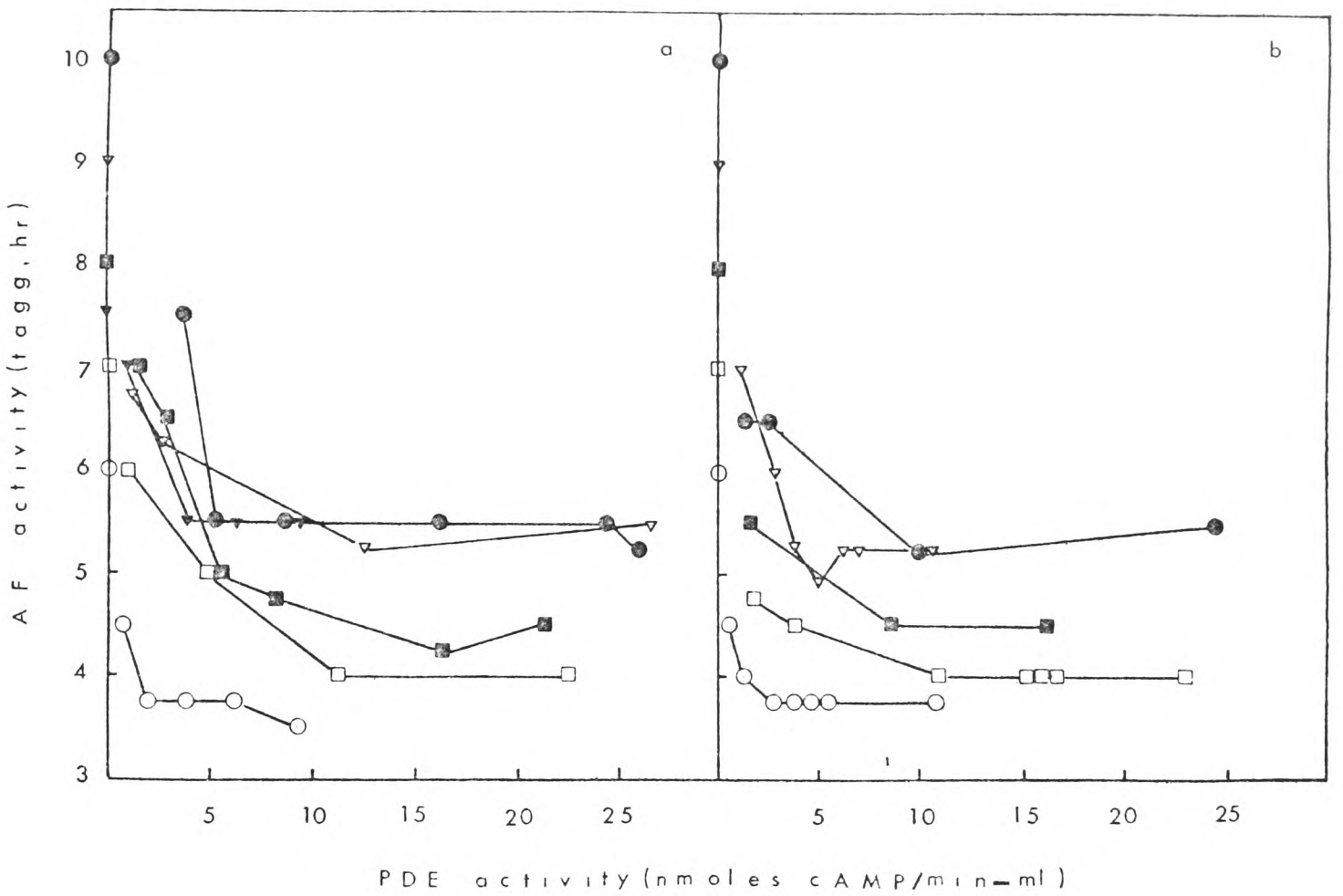

FIGURE 11

Plots of t_{agg} versus extracellular-PDE activity in (a) series of diluted crude Ax_2 t_2 supernatant (6 experiments) and (b) in series of partially inhibited extracellular PDE. The supernatant was diluted in KK2 buffer and the ammonium sulphate preparation of a heated t_{14} supernatant was used as the PDE inhibitor source. In one experiment (□) an inhibitor solution partially purified by DEAE cellulose chromatography was utilised. AF assays were performed in parallel for each two series with the same symbols. Each point represents the mean value of t_{agg} for each set of five replicas. Points in the ordinate represent the time of aggregation in the control (kk2 buffer). Maximum deviation from the mean t_{agg} did not exceed ½ hr.

control, seemed to require higher activities of PDE at t_o (approximately 5 units/ml) for full acceleration of interphase. To check if the decrease in AF activity after partial inhibition of PDE could be due to a toxic effect of the inhibitor, PDE activity was restored in these solutions by addition of a small volume of a 70 units/ml PDE preparation. As a control, an equal proportion of KK2 buffer was added to a second fraction of the same solution of partially inhibited PDE. The solution regained AF activity in the first case; t_{agg} remained unchanged in the control.

Crude or partially purified extracellular-PDE-inhibitor did not exhibit any AF activity and, in some cases, delayed onset of aggregation by 1-2 hours in relation to the KK2 control.

Conclusions

The secretion of AF into the extracellular medium during inter-phase has been demonstrated. It reduced t_{agg} to 45-65% of t_{agg} in the KK2 control.

AF is heat-labile and is precipitated by addition of ammonium sulphate to 60% saturation. AF activity is totally eluted in the void volume of Sephadex G 25 columns (fractionation range = 1,000-5,000 daltons) and is retained by ultrafiltration membranes with a nominal retentivity of 10,000 daltons. Therefore AF is not a small molecule.

Bovine serum albumin solutions failed to show any effect on duration of interphase; thus the AF effect is specific and is not merely a physico-chemical effect produced by extracellular proteins in general. On DEAE cellulose chromatography AF co-fractionated with the low Km form of extracellular PDE. This co-separation was maintained after gel-filtration on Sephadex G 200. Both activities were separated from a crude AF solution by electrophoresis on 7% polyacrylamide

gels and again AF activity coincided with PDE activity; a second and smaller (at 6 x 10^{-5}M cAMP) peak in PDE activity, not coinciding with AF activity, is interpreted as, possibly, corresponding to the high Km form of the enzyme which is known to be present in these supernatants (Chang, 1968; Chassy et al. 1969b; Pannbacker & Bravard, 1970; Chassy, 1972). These different methods of fractionation indicate that AF activity is related to extracellular PDE activity and that the two activities depend either on the same molecule or on closely associated molecules.

Inhibition or dilution of extracellular-PDE to an activity below 2.5 units/ml reduced AF activity. At 7.5 units/ml PDE activity or more AF activity was maximal. These results indicate that PDE activity is essential for AF activity. However, this evidence alone is not sufficient to justify the conclusion that AF activity and PDE activity are identical since the two activities might be related to different sites on the PDE molecule. (Inhibition of the PDE site might affect the second, AF site).

The experiments on rat brain PDE provide more definite evidence on the question of the identity of AF and PDE activities. Rat brain PDE (Km approximately 10^{-6}M) was assayed for AF activity and it proved to be as active as slime mould PDE in promoting acceleration of aggregation competence. In view of the evolutionary distance between rats and slime moulds, it is unlikely that a second, "AF" site has been conserved in the rat brain PDE, or that rat brain PDE and slime mould PDE have the same capacity to form complexes with other molecules. It is concluded that AF activity depends solely on cAMP hydrolysis, i.e. that AF activity and PDE activity are identical.

Experiments on the kinetics of changes in PDE and AF activities in the extracellular medium during interphase showed that when the cells were kept in suspension the early (t_2) peak in PDE activity might not correspond to maximal AF activity (reached only at t_5). Two interpretations of this phenomenon may be proposed without denying the correspondence between AF and PDE activities: (1) early supernatants may contain products which slightly retard AF activity; (2) early supernatants may contain a higher proportion of high Km form of extracellular PDE which seems to be inactive as AF. These questions are open to investigation.

2-3. Mode of action of AF (extracellular PDE)

2-3-1. The significance of extracellular PDE inhibition during interphase. In the previous sections the concept of AF (an extracellular soluble factor promoting acceleration of the pre-aggregation phase) was introduced. From several procedures of purification (including DEAE cellulose chromatography, gel filtration on Sephadex G 200 and electrophoresis in polyacrylamide gels) as well as from experiments with rat brain cAMP-phosphodiesterase and with partially inhibited PDE, it was concluded that AF corresponds to the low Km form of PDE. This finding contradicts the general belief that differentiation towards aggregation competence requires inhibition of extracellular PDE at an early phase of the pre-aggregation stage. Riedel & Gerisch (1971) suggested that inhibition of extracellular PDE allows the extracellular cAMP concentration to rise to levels promoting the cell shift from the vegetative to the developing state. This assumption is based on the existence of a natural inhibitor of extracellular PDE, secreted into the medium from early interphase and on the characteristics of aggregation

defective mutants which are also defective on regulation of extracellular PDE activity (Riedel et al. 1973). To further investigate the phenomenon of PDE inhibition and its role in development, Ax_2 cells were suspended at a density of 8×10^6 per ml of AF solution (PDE activity = 41 units/ml). The suspension was kept agitated at 160 rev. min. at 22°C and, at intervals of 1 hour, samples were assayed for aggregation competence (two 10 µl aliquots were placed on NN-agar plates and onset of aggregation was monitored by microscopic observation). The extracellular medium was collected from another fraction, taken at the same time, by centrifugation first at 260 g for 1 min and then at 10,000 g for 20 min at 4°C; PDE activity in the supernatant was also assayed.

The preliminary conclusions from these experiments indicate that development of aggregation competence may occur at extracellular PDE activities as high as 32-41 units/ml. In these experiments, aggregation competence in AF treated cells occurred at approximately t_3 when PDE activity was 32 units/ml, compared with a t_{agg} of 5 hours in the control (KK_2 treated cells). The procedure needs, however, some refinement in future experiments (e.g. determination of the proportion of enzyme corresponding to each Km value, control with cells developing on solid substrata). Nevertheless, the evidence drawn from the experiments described below on the effect of addition of cAMP at t_o to developing cells and on the results of variation of time of addition or removal of AF (extracellular PDE), already give support to the conclusion that there is no need for PDE inactivation during interphase.

2-3-2. Effects of cAMP, 5'AMP, adenosine or adenine on duration of interphase. Knowing that AF is probably extracellular PDE attempts were made to clarify whether its activity as AF is related to the decrease in cAMP concentration or, alternatively, to the accumulation of one or more of the cAMP products of digestion.

Addition of 10^{-6}M or 5 x 10^{-4}M 5'AMP, adenosine or adenine to KK2 buffer, used as suspending media in AF assays, did not cause acceleration or retardation of interphase in comparison to the KK2 control. 10^{-6}M cAMP was equally ineffective but 5 x 10^{-4}M cAMP caused a small delay in the onset of aggregation. Furthermore, in conditions where the AF solution promoted aggregation competence in 3.5 hours, addition of 5 x 10^{-4}M cAMP to AF increased the duration of interphase to 5.5 hours (t_{agg} in the KK2 control = 8 hours). Adenosine, adenine or 5'AMP at 5 x 10^{-4}M in AF solution did not affect t_{agg} as compared to the AF control.

2-3-3. AF as an extracellular factor of development. The presence of AF activity in rat brain PDE gave an indication that the activity of AF might be confined to the extracellular medium, i.e. AF might not be taken up or adsorbed by the cells. An attempt was made to immobilise AF for subsequent AF assays; persistance of AF activity in the insoluble form of PDE would also add further evidence to the previous conclusion on the nature of AF.

AF in three different solutions (crude t_{14} supernatant, crude t_2 supernatant and DEAE cellulose purified t_{14} supernatant) was coupled to Sepharose 4B (Pharmacia Fine Chemicals). These preparations were used for AF and PDE assays according to the procedure described in Methods (in this thesis). The final PDE activity in the different gel suspensions is indicated in brackets in Table 7.

Table 7

	t_{agg} (hr)		
	(a)	(b)	(c)
Insoluble t_{14}	6 (2.5)	5½ (1.25)	6 (1.25)
Soluble t_{14}	5½ (16)	3½ (16)	4¼ (16)
Insoluble t_2	5½ (4)	5½ (2)	6 (2)
Soluble t_2	6 (64)	4 (64)	4½ (64)
Insoluble F^+t_{14}	6½ (2.5)	5 (1.25)	5½ (1.25)
Soluble F^+t_{14}	6 (20)	4 (20)	4½ (20)
KK2 buffer	8½ (0)	7½ (0)	9½ (0)

AF activity of insolubilised AF. Activity in Sepharose-bound AF is compared to activity in the corresponding soluble forms. PDE activity (units/ml) is indicated in brackets. Data from three experiments is presented; in experiments (b) and (c) the gel suspensions were diluted 2-fold in KK2 buffer in order to facilitate microscopic observation. The supernatants of AF gels and a control gel (inactivated Sepharose 4B) produced t_{agg} values similar to t_{agg} in the KK2 control.

F^+, AF in DEAE fractionated t_{14} supernatant.

In experiment (a), where a high density of beads was used, the AF activity in soluble and insoluble AF was identical. In experiments (b) and (c) the gel suspensions were diluted by a factor of 2 to facilitate the detection of onset of aggregation in the bead-intermingled cell populations. Although t_{agg} in these preparations, was increased in relation to the soluble form of AF it was still markedly different from the value for the KK2 control. Supernatants of AF gels and the control gel (inactivated Sepharose 4B) failed to produce any acceleration of interphase.

Conclusions

Ribose mononucleotides have been reported (Krichevsky et al. 1969) to accelerate the rate of development toward formation of fruiting bodies when at concentrations of 10^{-4} M or more. However, during the pre-aggregation stage 5'AMP as well as adenosine or adenine at concentrations of 5×10^{-4} M or 10^{-6} M did not produce any change in the timing of aggregation competence. In addition AF proved to have an exclusively extracellular function. This, in conjunction with the accumulated evidence on identity of AF and extracellular PDE (section 2-2) and with the observation that cAMP added at t_o may retard AF activity, leads to the conclusion that AF acts by lowering extracellular cAMP concentration during interphase.

Evidence here is against the proposal that inhibition of extracellular PDE during interphase is required for cell differentiation.

2-4. AF as a control of rate of development toward aggregation competence.

2-4-1. AF and the induction of competence for chemotaxis. To distinguish between the possibility of AF (PDE) being indispensable

or stimulatory at the time of aggregation (e.g. if AF is required for effective signal propagation) and the alternative hypothesis that AF is involved in the process of differentiation toward aggregation competence, experiments were made to determine the time of appearance of a cell property characteristic of the pre-aggregation stage in conditions of presence and absence of AF. The capacity for chemotactic response towards cAMP is known to increase spectacularly during interphase (Shaffer, 1957 a; Bonner, et al. 1969). It was reasoned that if the effect of AF was exerted only in late interphase or during aggregation itself then the rate of development of chemotactic ability would be similar in AF and KK2 buffer treated cells. On the other hand if the rate of differentiation was accelerated by AF since early interphase, both chemotaxis and the ability for stream formation (which involves increase in cAMP receptors, capacity for signal amplification and formation of EDTA-resistant cell contacts) would develop earlier in AF treated cells than in controls. As a preliminary experiment, sparse populations of Ax_2 cells were tested after 8 hours of interphase for response to an artificial source of cAMP (10^{-5}M solution). The result was negative even when the cells were harvested from the original NN-agar plates and concentrated just before being challenged with cAMP. Conversely cells developing early aggregation-competence at high density would respond to cAMP even when spread at low density on NN-agar plates. A closer study of the development of chemotactic response in AF and KK2 buffer-treated cells was therefore undertaken.

Ax_2 cells suspended at t_o in AF solution or KK2 buffer at a density of 7×10^6/ml were tested hourly for chemotactic ability toward 10^{-4}M and 10^{-5}M cAMP solutions placed, in 5 µl volumes, at

Table 8

Extracellular medium	Chemotaxis		Spontaneous aggregation
	Separate cells (hr)	Streams (hr)	t_{agg} (hr)
$AF_{(a)}$	4	5½	6
KK2	8	8-9	10
$AF_{(a)}$	4-5	5-6	6
KK2	8-9	8-10	10½
$AF_{(b)}$	3½-5	5½	5½
KK2	8-9	9	9½

Response to an artificial source of cAMP by AF and KK2 buffer treated cells (3 experiments). At t_o several 10 µl aliquots of suspensions at 7×10^6 cells per ml of KK2 buffer or AF solution were deposited on NN-agar plates and incubated. At intervals of one hour, sample populations were tested for response toward sources of cAMP (10^{-4}M solution) placed at approximately 1 mm distance from their periphery. The time of chemotactic response as separate cells and with formation of streams is indicated as well as t_{agg} in non-challenged populations. (a) cells suspended in 2 x concentrated t_{14} supernatant (44 units/ml PDE); (b) cells suspended in t_2 supernatant (20 units/ml PDE).

approximately 1 mm from the periphery of the test populations. Table 8 shows the results obtained with the 10^{-4}M cAMP solution in terms of development of simple chemotactic ability (movement as separate cells) and ability for stream formation. In the KK2 buffer controls chemotaxis was never clear, in these conditions, before t_8 but AF treated cells showed a marked response from about t_4 or t_5. Ability to form streams developed, in both cases, very close to the time of spontaneous aggregation (t_{agg}) which differed in AF and control populations by 3½ to 4 hours. 10^{-5}M cAMP solutions elicited similar results but the range of cAMP influence was decreased.

2-4-2. Development of aggregation competence as a function of time of AF addition; reversibility of the early period of differentiation. From the results on the development of chemotaxis toward cAMP (section above) it was concluded that AF (PDE) is active during interphase. It was, then, of interest to define more precisely the period of AF activity. Ax_2 cells were suspended at a density of 5-7 x 10^6/ml in KK2 buffer. The suspension was either kept agitated at 160 rev.p.min (Fig. 12 a, b) or was spread on NN-agar plates (Fig. 12 c, d) at 0.5 ml of cell suspension per plate. Incubation was at 22°C, with illumination, the NN-agar plates being kept in humidity chambers. At hourly intervals during interphase (t_o inclusive), two aliquots of 100 µl each, taken directly from the agitated suspension or from the agar preparations (after gentle re-spreading with a glass rod; distinct cell preparations were used each time to avoid repeated re-spreading) were centrifuged in 1 ml Duran tubes at 260 g for 1 min. KK2 buffer or recently thawed AF solution, both kept in an ice-bath, were substituted for

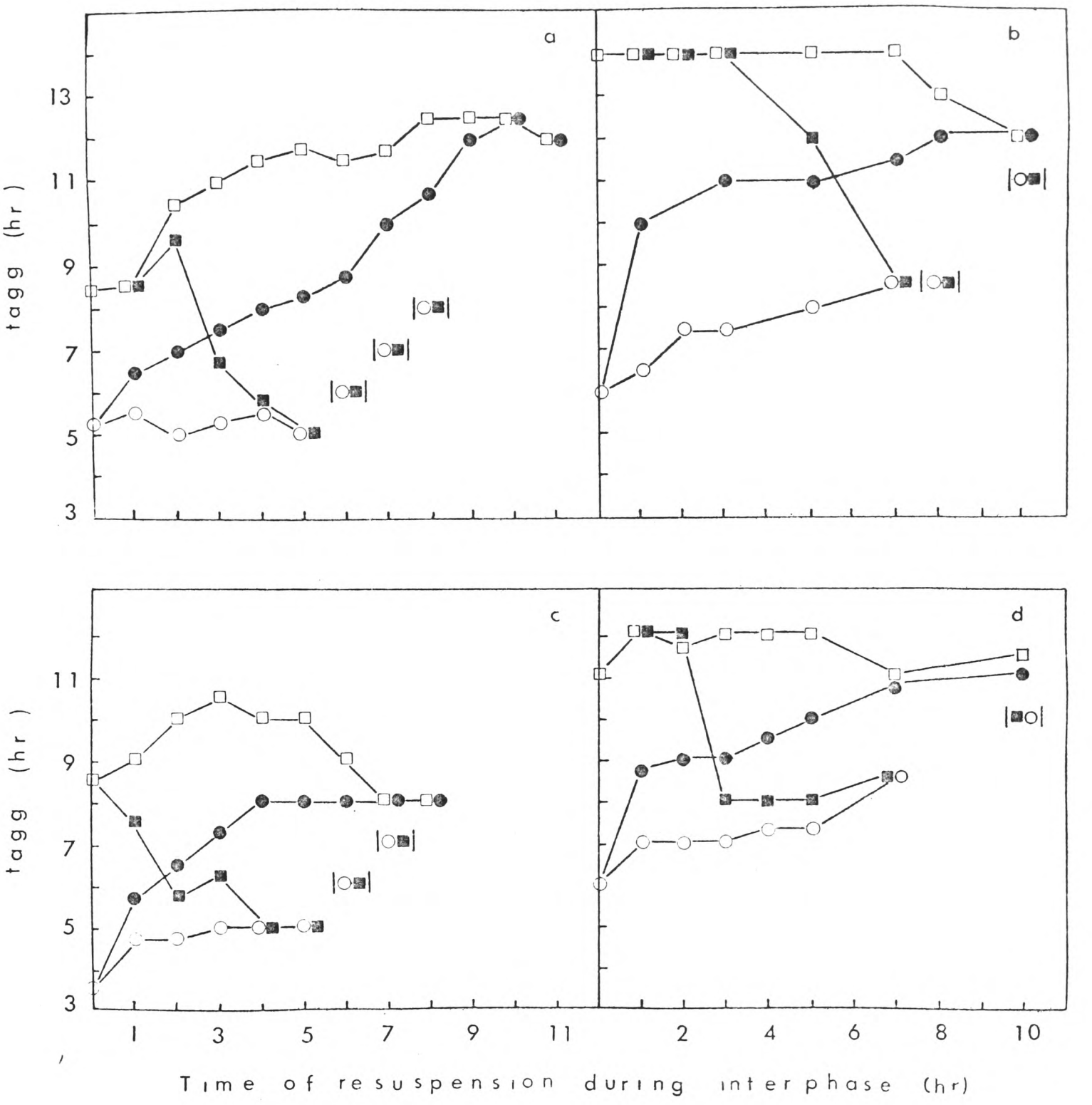

FIGURE 12

Period of action of AF. Four experiments were conducted in which Ax_2 cells suspended at t_o in KK2 buffer and AF solutions were kept either in suspension with agitation (a,b) or were spread over NN-agar plates (c,d). At different times of interphase (■) the AF solution was replaced by KK2 buffer or (●) KK2 buffer was replaced by AF solution in aliquots taken from the initial cell suspensions. Controls were run in the same conditions but the same type of medium used for pre-treatment was utilised for resuspension: (o) AF pre-treated cells resuspended in AF, (□) KK2 buffer pre-treated cells resuspended in KK2 buffer. The time of aggregation (t_{agg}) was monitored by microscopic observation of 10 µl populations deposited on NN-agar at the time of resuspension. Each point represents the mean t_{agg} for each set of five replicas, the variation from the mean not exceeding ¼ hour. Points enclosed in bars denote immediate re-aggregation. PDE activity in the AF solutions: 10 units/ml (a,b), 13 units/ml (d) and 20 units/ml (c).

the supernatant after centrifugation. After 10 seconds vortex mixing, 10 µl drops (five replicas) of cell suspension were deposited on NN-agar plates and incubated for further development. The time of onset of aggregation (t_{agg}) was monitored by regular microscopic observation. This experiment was intended to time the initiation of the period of sensitivity to AF.

In order to get information on the stability of the process of differentiation and on the total duration of the period of sensitivity to AF, similar experiments were carried out, concurrently with the ones described above, with cells suspended at t_o in AF solution. At hourly intervals during interphase, new (freshly thawed) AF solution or KK2 buffer substituted for the extracellular medium. Fig. 12 shows the results of the two sets of experiments. At this cell density (5-7 x 10^6 cells/ml) the conditions of incubation during pre-treatment (shaken suspensions or preparations on agar) did not appear to have any marked effect on the overall results. Delays in the addition of AF during interphase resulted in decreased AF activities (measured as the difference in t_{agg} in relation to the KK2 buffer control). The sensitivity to AF was specially marked during the first hour of interphase and decreased, progressively, with time. At the aggregation competence stage AF became completely dispensable (points within bars). This indicates a critical action of AF between t_o and t_1 although its activity was still perceptible at the late pre-aggregation stage. However, after removal of AF at early interphase (t_1 - t_3) no signs of earlier initiation of differentiation or faster development were found. This period of reversible differentiation was somehow decreased in a fast developing population (Fig. 12 c).

Conclusions

AF (PDE) affects the rate of development toward aggregation competence. This is reflected in the accelerated development of increased sensitivity to cAMP and in the capacity for earlier formation of streams by AF-treated cells when compared to KK2 buffer treated cells. Although no quantitative assays on development of cAMP receptors or on development of contact sites A (usually taken as indicators of rate of development in interphase) were carried out, the results presented already predict an increase in the rate of their appearance at the cell surface. The early sensitivity (from t_o) to AF indicates that differentiation may start as early as t_o. The early period of differentiation was found to be reversible after removal of AF. Darmon and co-workers (1975) obtained similar results when the development of AX_2 cells toward aggregation competence was accelerated by application of cAMP pulses.

The evidence accumulated supports the proposal that cAMP oscillations control the rate of development (Gerisch et al, 1975 a; Darmon et al, 1975). Since extracellular PDE acts from the beginning of interphase (t_o), cAMP oscillations may also occur from early interphase at least in the presence of this enzyme. This would imply that the cAMP oscillator is a determinant, but not a product, of the developmental programme.

3. Morphogenesis and the duration of the pre-aggregation stage.

The AF promoted decrease in the duration of the pre-aggregation stage could reflect an acceleration of the whole of an integrated process of differentiation toward the ultimate formation of a fruiting body; alternatively, this process of differentiation

might include parallel programmes some of which might not be affected by AF. In the latter case one could predict either a lag in post-aggregation development or an impaired late development. This possibility was tested in five experiments in which AF and KK2 buffer treated Ax_2 cells were either spread on NN-agar plates or deposited as spot-populations (10 µl of cell suspension) on similar plates. Development took place in humidity chambers and in the light. AF treated cells showed a t_{agg} varying, in separate experiments, from 4 to 6 hours compared to t_{agg} values in the KK2 control of 8 to 10 hours. "Conus" appeared, respectively, at t_8-t_{10} and t_{12}-t_{14}. This stage was followed by the formation of "slugs" after a period of approximately two hours. Fruiting bodies were formed first in AF treated populations, 19 hours after the initiation of development (compared to about 24 hours in the KK2 buffer control). The early advancement of development was always reflected in a corresponding advancement in the formation of fruiting bodies and of all the intermediate stages. Development follows thus an integrated process of differentiation.

APPENDIX 1

Strain	Cell density (cells/cm2)	Movement duration (X) (sec)	$\bar{X}-X$	$(\bar{X}-X)^2$
NC4	5×10^5	120	-20.34	413.72
		100	- 0.34	0.12
		96	3.66	13.40
		108	- 8.34	69.56
		108	- 8.34	69.56
		108	- 8.34	69.56
NC4	5×10^5	104	- 4.34	18.84
		96	3.66	13.40
		68	31.66	1002.36
NC4	5×10^5	88	11.66	135.96
		88	11.66	135.96
NC4	3×10^5	112	-12.34	152.28
		100	- 0.34	0.12
NC4	1.5×10^5	80	19.66	386.52
		108	- 8.34	69.56
		96	3.66	13.40
		112	-12.34	152.28
NC4	1.25×10^5	116	-16.34	266.99
		100	- 0.34	0.12
		88	11.66	135.96
		96	3.66	13.40
Ax_2	5×10^5	100	- 0.34	0.12
		76	23.66	559.80
		124	-24.34	592.44

Number of observations (N) = 24

Mean movement duration $(\bar{X}) = \frac{2392}{24} = 99.66$ sec. $\quad \Sigma(\bar{X}-X)^2 = 4285.43$

Standard deviation(s) $= \sqrt{\frac{\Sigma(\bar{X}-X)^2}{N-1}} = \pm 13.65$

APPENDIX 2 (Table 4)

Intercellular distance	Velocity (Y)	Number of observations (n)	Mean veloc. $\bar{Y}$	$\bar{Y}$-Y	$(\bar{Y}-Y)^2$	SEM+
51	7.05	2	6.89	-0.16	0.0256	±0.67
51	6.58	1		0.38	0.0961	
51	7.24	1		-0.94	0.88	±0.42
51	6.30	4	6.30	0.00	0.00	
51	5.83	2		0.47	0.22	
51	5.17	1		0.47	0.22	
51	5.64	3	5.64	0.00	0.00	±0.41
51	6.11	1		-0.47	0.22	
51	5.34	7		0.13	0.17	
51	6.40	5		-0.93	0.86	
			5.47			±0.39
51	4.37	3		1.10	1.21	
51	4.95	1		0.52	0.27	
32	5.35	18		0.42	0.18	
32	5.85	7		-0.08	0.01	
32	6.30	10		-0.53	0.28	
			5.77			±0.18
32	6.75	1		-0.98	0.96	
32	7.20	3		-1.43	2.04	
32	4.89	4		0.88	0.77	
32	5.86	5		-0.13	0.02	
			5.23			±0.20
32	4.89	2		0.34	0.12	

Appendix 2 (Table 4) Continued

Intercellular distance	Velocity (Y)	Number of observations (n)	Mean veloc. ($\bar{Y}$)	$\bar{Y}$-Y	$(\bar{Y}-Y)^2$	SEM$^+$
29	5.64	1		0.80	0.64	
29	6.11	1		0.33	0.11	
			6.44			±0.40
29	6.57	4		-0.13	0.02	
29	7.04	1		-0.60	0.36	
29	4.70	9		0.05	0.00	
			4.75			±0.10
29	5.17	1		-0.42	0.18	
29	4.70	4	4.70	0.00	0.00	-
26	4.95	5		0.33	0.11	
26	5.36	4		-0.08	0.01	
			5.28			±0.27
26	5.63	1		-0.35	0.13.	
26	6.30	1		-1.02	1.04	
22.5	6.30	3		0.18	0.03	
22.5	5.83	2		0.65	0.42	
			6.48			±0.47
22.5	7.24	2		-0.76	0.58	
22.5	6.77	1		-0.29	0.08	

Appendix 2 (Table 4) Continued

Intercellular distance	Velocity (Y)	Number of observations (n)	Mean veloc. ($\bar{Y}$)	$\bar{Y}$-Y	$(\bar{Y}-Y)^2$	SEM+
22.5	4.90	20		0.73	0.53	
22.5	5.36	19		0.27	0.07	
22.5	5.83	11		-0.20	0.04	
			5.63			±0.16
22.5	6.30	9		-0.67	0.45	
22.5	6.77	6		-1.14	1.30	
22.5	7.24	3		-1.61	2.60	
22.5	4.70	7		0.06	0.00	
			4.76			±0.14
22.5	5.17	1		-0.41	0.17	
16	5.38	3		0.13	0.02	
16	5.60	1		-0.09	0.01	
			5.51			±0.28
16	5.36	3		0.15	0.02	
16	4.89	1		0.62	0.38	
16	4.70	5	4.70	0.00	0.00	-
16	4.70	5	4.70	0.00	0.00	-

+The standard error (SEM) was calculated (Bailey, 1972) for 95% confidence limits by introducing a factor t corresponding to N-1 degrees of freedom).

$$SEM = s/\sqrt{N}$$

$$s = \sqrt{\Sigma(\bar{Y}-Y)^2/N-1}$$

$$N = \Sigma n$$

APPENDIX 3 (Table 4)

Intercellular distance (X)	Signal velocity (y)	Number of observations (n)	X^2	Y^2	XxY
51	7.05	2	2601	49.70	359.55
51	6.58	1	2601	43.29	335.58
51	7.24	1	2601	52.41	369.24
51	6.30	4	2601	39.69	321.30
51	5.83	2	2601	33.98	297.33
51	5.34	7	2601	28.51	272.34
51	6.40	5	2601	40.96	326.40
51	4.37	3	2601	19.09	222.87
51	4.95	1	2601	24.50	252.45
51	5.17	1	2601	26.72	263.67
51	5.64	3	2601	31.80	287.64
51	6.11	1	2601	37.33	311.61
32	5.35	18	1024	28.62	171.20
32	5.85	7	1024	34.22	187.20
32	6.30	10	1024	39.69	201.60
32	6.75	1	1024	45.56	216.00
32	7.20	3	1024	51.84	230.40
32	4.89	4	1024	23.91	156.48
32	5.36	5	1024	28.72	171.52
32	4.89	2	1024	23.91	156.48
29	4.70	9	841	22.09	136.30
29	5.17	1	841	26.72	149.93
29	5.64	1	841	31.80	163.56
29	6.11	1	841	37.33	177.19
29	6.57	4	841	43.16	190.53
29	7.04	1	841	49.56	204.16
29	4.70	4	841	22.09	136.30
26	4.95	5	676	24.50	128.70
26	5.36	4	676	28.72	139.36
26	5.63	1	676	31.69	146.38
26	6.30	1	676	39.69	163.80

APPENDIX 3 (Table 4) Continued

Intercellular distance (X)	Signal velocity (Y)	Number of observations (n)	X^2	Y^2	XxY
22.5	6.30	3	506.25	39.69	141.75
22.5	5.83	2	506.25	33.98	131.17
22.5	7.24	2	506.25	52.41	162.90
22.5	6.77	1	506.25	45.83	152.32
22.5	4.70	7	506.25	22.09	105.75
22.5	5.17	1	506.25	26.72	116.32
22.5	4.90	20	506.25	24.01	110.25
22.5	5.36	19	506.25	28.72	120.60
22.5	5.83	11	506.25	33.98	131.17
22.5	6.30	9	506.25	39.69	141.75
22.5	6.77	6	506.25	45.83	152.32
22.5	7.24	3	506.25	52.41	162.90
16	5.83	3	256	33.98	93.28
16	5.60	1	256	31.36	89.60
16	5.36	3	256	28.72	85.76
16	4.89	1	256	23.91	78.24
16	4.70	5	256	22.09	75.20
16	4.70	5	256	22.09	75.20

$N = \Sigma n = 215$ $\quad \Sigma Y^2 = 6793.512$

$\Sigma X = 6254$ $\quad (\Sigma X)^2 = 39112516$

$\Sigma Y = 1198.57$ $\quad (\Sigma Y)^2 = 1436570.04$

$\Sigma X^2 = 204061$ $\quad \Sigma XY = 35150.065$

Degree of association between intercellular distances and signal velocity (From Bailey, 1972).

$$\text{Correlation coefficient}(r) = \frac{\Sigma XY - \frac{1}{N}\Sigma X.\Sigma Y}{\sqrt{\left(\Sigma X^2 - \frac{(\Sigma X)^2}{N}\right)\left(\Sigma Y^2 - \frac{(\Sigma Y)^2}{N}\right)}} = \pm 0.082$$

Significance of the correlation coefficient:

t (student's t) $= r\sqrt{\frac{N-2}{1-r^2}} = \pm 2.70$; the correlation coefficient is significantly different from zero ($0.02 > P > 0.01$).

APPENDIX 4 (Table 2)

Intercellular distance (X)	Velocity (Y)	X^2	Y^2	XxY
32.0	5.9	1024.00	34.81	188.8
32.0	5.1	1024.00	26.01	163.2
29.0	4.4	841.00	19.36	127.6
29.0	5.7	841.00	32.49	165.3
29.0	4.1	841.00	16.81	118.9
29.0	4.3	841.00	18.49	124.7
27.0	4.9	729.00	24.01	132.3
27.0	4.5	729.00	20.25	121.5
27.0	4.5	729.00	20.25	121.5
27.0	4.8	729.00	23.04	129.6
27.0	4.7	729.00	20.09	126.9
27.0	5.2	729.00	27.04	140.4
22.5	3.5	506.25	12.25	78.8
22.5	4.4	506.25	19.36	99.0
22.5	3.6	506.25	12.96	81.0
22.5	4.3	506.25	18.49	96.8
22.5	3.6	506.25	12.96	81.0
22.5	4.3	506.25	18.49	96.8
16.0	4.2	256.00	17.64	67.2
16.0	4.3	256.00	18.49	68.8
16.0	3.6	256.00	12.96	57.6
16.0	3.8	256.00	12.96	60.8

APPENDIX 4 (Table 2) continued

$\Sigma X = 541.00$ $\quad \Sigma X^2 = 13847.5$

$\Sigma Y = 97.7$ $\quad \Sigma Y^2 = 442.69$

$\Sigma XY = 2448.5$

$(\Sigma X)^2 = 292681$ $\quad N = 22$

$(\Sigma Y)^2 = 9506.25$

$\Sigma X . \Sigma Y = 52855.5$

Degree of association between intercellular distance and signal velocity (from Bailey, 1972)

$$\text{Correlation coefficient (r)} = \frac{\Sigma XY - \frac{1}{N}\Sigma X . \Sigma Y}{\sqrt{\left(\Sigma X^2 - \frac{(\Sigma X)^2}{N}\right)\left(\Sigma Y^2 - \frac{(\Sigma Y)^2}{N}\right)}} = \pm 0.60$$

Significance of the correlation coefficient:

t (student's t) $= r\sqrt{\frac{N-2}{1-r^2}} = \pm 3.35$; the correlation coefficient is significantly different from zero ($0.01 > P > 0.002$)

APPENDIX 5 (Table 3)

Intercellular distance (X)	Velocity (Y)	X^2	Y^2	XxY
51	8.9	2601	79.21	453.9
51	8.0	2601	64.00	408.0
51	7.1	2601	50.41	362.1
51	4.4	2601	19.36	224.4
51	4.2	2601	17.64	214.2
32	7.2	1024	51.84	230.4
32	5.4	1024	29.16	172.8
32	5.8	1024	33.64	185.6
32	4.3	1024	18.49	137.6
32	4.6	1024	21.16	147.2
32	8.3	1024	68.89	265.6
29	5.6	841	31.36	162.4
29	4.8	841	23.04	139.2
29	5.8	841	33.64	168.2
29	4.5	841	20.25	130.5
29	5.4	841	29.16	156.6
29	6.8	841	46.24	197.2
29	5.5	841	30.25	159.5
29	5.9	841	34.81	171.1
29	5.5	841	30.25	159.5
29	5.1	841	26.01	147.9
25	3.8	625	14.44	95.0
25	3.5	625	12.25	87.5
18	4.0	324	16.00	72.0
18	3.1	324	9.61	55.8

APPENDIX 5 (Table 3) Continued

Intercellular distance (X)	Velocity (Y)	X^2	Y^2	XxY
18	4.9	324	24.01	88.2
18	3.6	324	12.96	64.8
18	3.3	324	10.89	59.4
18	6.2	324	38.44	111.6
16	3.3	256	10.89	52.8
16	3.7	256	13.69	59.2

$\Sigma X = 927$ $\quad \Sigma X^2 = 31265$

$\Sigma Y = 162.5$ $\quad \Sigma Y^2 = 921.99$

$(\Sigma X)^2 = 859329$ $\quad \Sigma XY = 5140.2$

$(\Sigma Y)^2 = 26406.25$ $\quad N = 31$

$\Sigma X.\Sigma Y = 150637.5$

Degree of association between intercellular distance and signal velocity (from Bailey, 1972)

$$\text{Correlation coefficient } (r) = \frac{\Sigma XY - \frac{1}{N}\ \Sigma X.\Sigma Y}{\sqrt{(\Sigma X^2 - \frac{(\Sigma X)^2}{N})\ \ (\Sigma Y^2 - \frac{(\Sigma Y)^2}{N})}} = \pm 0.56$$

significance of the correlation coefficient:

$$t \text{ (student's t)} = r\sqrt{\frac{N-2}{1-r^2}} = \pm\ 3.64;$$ the correlation is significantly different from zero ($0.001 < P < 0.002$)

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Signal Propagation during Aggregation in the Slime Mould *Dictyostelium discoideum*

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SUMMARY

We have analysed the pattern of concentric waves visible at the onset of aggregation of amoebae of *Dictyostelium discoideum* and have shown that each wave consists of a light band of elongated, moving cells, and a darker interband zone of rounded cells. Our analysis supports earlier suggestions that aggregation occurs in response to chemotactic signals emanating from a centre and which are propagated outwards through the field by a relay mechanism. The width of each band of moving cells corresponds to the distance the signal is propagated during the time that the cells remain elongated after stimulation, and does not vary with signal frequency. The width of the interbands of non-moving cells depends upon the distance the signal is propagated *between* signalling events, and varies with the signal frequency which increases during aggregation. The velocity of signal propagation decreases slightly with increase in the density of the monolayer of aggregating cells. We have shown by time-lapse films taken at high magnification that the signal is relayed radially outwards in steps of approximately 57 μm (relay zones) and that the response of each successive zone occurs approximately 12 s after the previous one (relay time). We have attempted to demonstrate the existence of a refractory period for chemotactic responsiveness. Our results indicate that such a refractory period, if it exists, cannot be more than 12 s.

INTRODUCTION

In the life cycle of the cellular slime mould *Dictyostelium discoideum*, growth and differentiation occur separately. Unicellular amoebae feed and divide as long as food bacteria are present. When the bacteria are removed, the amoebae enter a period called interphase during which they develop the ability to aggregate at a central collecting point. The multicellular structure formed as a result of aggregation ultimately develops into a fruiting body from which spores may be dispersed to germinate as unicellular feeding amoebae (see reviews by Raper, 1940; Shaffer, 1962; Bonner, 1967; Gerisch, 1968; Newell, 1971).

Aggregation occurs by chemotaxis (Bonner, 1947). Signalling amoebae emit an attractant, called acrasin by Bonner (1947), which is probably cyclic AMP (Barkley, 1969; Konijn, Barkley, Chang & Bonner, 1968). Amoebae within range of a sufficient concentration of the diffusing signal respond by movement towards the signalling 'centre' cell(s). The responding cells show periodic movement steps suggesting that signal emission is itself periodic (Shaffer, 1962; Gerisch, 1965). The periodicity of response decreases during aggregation (Arndt's film, cited in Shaffer, 1962; Durston, 1974). Responding cells move towards each other and form streams moving towards the centre. The streams themselves also produce acrasin (Bonner, 1949; Shaffer, 1957*b*). These observations suggest that signalled amoebae respond not only by an inward movement step, but also by relaying the signal (Shaffer, 1957*b*),

perhaps again in the form of cyclic AMP. Thus there need not exist a steady gradient in concentration of acrasin from the centre outward; rather signalling 'secretory fronts' are propagated out by relay through the aggregation territory (Shaffer, 1957*b*; Gerisch, 1968; Cohen & Robertson, 1971*a*).

Unidirectional propagation of the signal, and centripetal cell movement, may require that cells become refractory for some time after each stimulation so as to prevent them from responding to acrasin released in turn by more distal cells (Gerisch, 1965; Cohen & Robertson, 1971*a*). They would have to remain refractory at least until the signal released distal to them is reduced to below threshold levels. This reduction is thought to be accomplished by inactivation of the attractant (Shaffer, 1957*b*). If this is cyclic AMP, inactivation would be brought about by the phosphodiesterase bound to the cell membrane and released into the medium (Chang, 1968; Malchow, Nägele, Schwarz & Gerisch, 1972; Pannbacker & Bravard, 1972; Malkinson & Ashworth, 1973). The phosphodiesterase, in destroying the cyclic AMP, would also permit detection of the next signal propagated from the centre.

Waves of inward movement spreading out from a signalling centre have been demonstrated by time-lapse films (Bonner, 1944, cited in Bonner, 1967). Moreover, early in aggregation, characteristic patterns of concentric bands may be visible (Gerisch, 1965). This paper reports an analysis of this band pattern and shows the individual relay events involved in signal propagation. We have also attempted to demonstrate the existence of a refractory period for chemotactic responsiveness.

METHODS

Strains. Dictyostelium discoideum strain NC4, the axenic strain AX2, and the bacterial associate, *Aerobacter aerogenes*, were kindly provided by Dr J. Ashworth.

Media. KK_2 buffer contained (g/l water): KH_2PO_4, 2·25; K_2HPO_4, 0·67; $MgSO_4.7H_2O$, 0·5; pH 6·1. Standard medium, SM (Sussman, 1951) contained (g/l KK_2 buffer): Bacto-peptone (Difco), 5; yeast extract (Difco), 0·5. For SM agar, 20 g Bacto-agar (Difco) were added to 1 l SM broth. After autoclaving (15 lb/in^2, 15 min), sterile glucose was added to SM broth and SM agar to 0·5% final concentration. Axenic broth, HL5 (Watts & Ashworth, 1970) contained (g/l water): bacteriological peptone (Oxoid), 14·3; yeast extract (Difco), 7·15; $Na_2HPO_4.12H_2O$, 1·28; KH_2PO_4, 0·49; D-glucose, 15·4; pH 6·7. Non-nutrient (NN) agar, contained: Bacto-agar (Difco), 10 g; KK_2 buffer, 1 l.

Growth conditions. Strain NC4 was grown on SM plates in association with *A. aerogenes.* Fifteen spore heads (approx. 3×10^6 spores) and 0·2 ml of an overnight bacterial culture were spread over the surface of each plate. Vegetative amoebae were harvested after 24 h incubation at 22 °C, and the bacteria removed by four centrifugations at 400 *g* for 1 min, resuspending the amoebae each time in ice-cold KK_2.

Strain AX2 was grown in liquid culture (in 10 ml HL5 in a 100 ml Erlenmeyer flask) at 22 °C on a rotary shaker (150 rev./min.). The amoebae were harvested in exponential phase (5×10^6/ml), washed by centrifugation, and resuspended in ice-cold KK_2.

Cell densities were determined in a Petroff–Hauser counting chamber and adjusted by dilution as required, before the cells were transferred to agar or coverslip for aggregation.

Aggregation. Amoeba suspension (0·5 ml) was spread over the surface of fresh NN agar plates (5 ml NN agar in $8 \times 5 \times 1{\cdot}8$ cm plastic boxes). The amoebae were allowed to settle and disperse for 30 min and the agar surface drained of excess liquid. Partial evaporation of the residual liquid film was allowed by leaving the boxes open for 20 min. Our technique regularly produced fields of amoebae of uniform density.

For AX2, aggregation could also be followed on a glass coverslip. The amoeba suspension (0·01 ml) was spotted on to an alcohol-washed coverslip, the amoebae were allowed to settle for 5 min, and then washed free of growth medium by immersion of the coverslip in KK_2 buffer. Excess liquid was drained and the coverslip then inverted over a humidity chamber. This chamber had been prepared by fixing a clean coverslip over a hole drilled in a plastic microscope slide and then partially filling the hole with 10% (w/v) gelatin. The upper coverslip, bearing the amoebae, was sealed to the slide with paraffin. The time required for aggregation depends on the density of amoebae in the monolayer and the volume of liquid remaining on the coverslip after draining. These conditions were carefully controlled.

The preparations developing aggregation competence were incubated at 22 °C. Aggregation commenced at times ranging from 6 h for 5×10^5 amoebae/cm^2 to 13 h for 5×10^4 amoebae/cm^2. High-density preparations were sometimes kept at 7 °C for 18 h and then incubated at 22 °C until aggregation commenced. This took place within 1 h. The wave patterns obtained by both methods were similar.

Cell densities were checked by counting cells in the field under study, using calibrated eyepiece graticules, and confirmed in the films and photographs used in the analyses presented in this paper. We were careful to ensure that the fields studied were uniform in density.

The intercellular distance was calculated as $2/\sqrt{(\pi N)}$, where N is the cell density (Cohen & Robertson, 1971*a*). In this formula we approximate to a uniform distribution of amoebae, in which each cell is equidistant from its neighbours, by regarding the field as a network of contiguous circles centred on the individual cells. The diameter of the circles then corresponds to the distance between the *centres* of neighbouring cells.

Optical methods. A Wild M20 microscope was used for phase-contrast observation. It was equipped with a humidity chamber fitting both the slide carrier and the boxes used for aggregation. Fogging in the objectives was avoided by a fitted warming sleeve which raised the temperature in the objective by not more than 0·5 °C. A Bolex H16 Reflex cine-camera and a Paillard/Wild Variotimer were used for time-lapse films, which were taken at a speed of 1 frame/4 s and analysed frame by frame, or in sets of frames, in a Litax film analyser. Photographs were taken with a Rada roll-film adaptor. Films and photographs were calibrated by filming or photographing a micrometer at the same magnification as used for the experimental material. For direct measurements under the microscope a calibrated eyepiece was used.

RESULTS

The concentric wave pattern

Territories exhibiting concentric wave patterns during the early stages of aggregation are shown in Fig. 1. Provided the amoebae are spread evenly and the surface moisture is controlled (see Methods) we consistently observed these patterns in populations of amoebae spread on the surface of buffered agar at densities ranging from 10^5 amoebae/cm^2 to 5×10^5 amoebae/cm^2 (an almost confluent monolayer). We used 1% rather than 2% (w/v) agar, for better optical definition. Gerisch (1965) published similar photographs of wave patterns that arose when amoebae were spread in dense multilayers. Microscopical examination of fields such as those in Fig. 1 showed that at any instant the light bands consisted of elongated moving cells, while the darker interband areas contained rounded cells exhibiting randomly-oriented pseudopodia. Contiguous areas of the two cell forms are seen in Fig. 2. The concentric wave pattern may easily be understood if each band of moving cells corresponds to the zone of influence, at a given time, of a wave of stimulation propagating outwards from the centre of the aggregation field. The darker interband areas would then contain cells

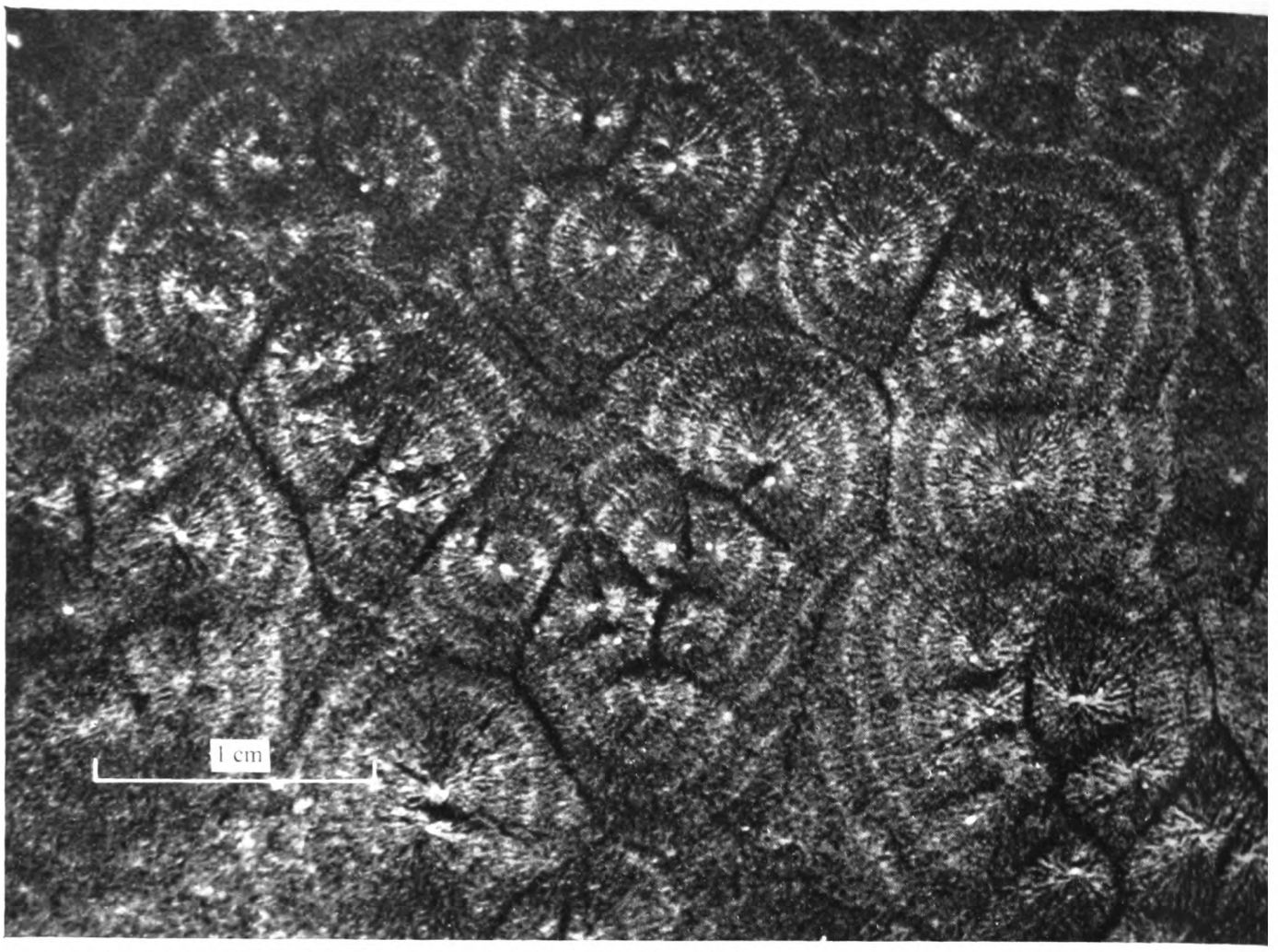

Fig. 1. The concentric wave pattern in fields of *D. discoideum* NC4 aggregating on the surface of NN agar (cell density 10^5 amoebae/cm^2). The light bands are bands of moving cells. Some spiral wave patterns are also present (see Gerisch, 1971). The photograph (by J. Kinross) was obtained with a camera fitted with a 4×5 in sheet film back and Ilford FP4 film (exposure 1/8 s at $f = 4{\cdot}7$). A Kodak cold light illuminator and a 180 mm condenser were set to provide a form of dark-field illumination.

which have ceased to move in response to a signal that has just passed them and which have not yet been stimulated by the next signal. This explanation was confirmed by examining the same group of cells for some time, either directly or in time-lapse films. The cells alternated between the elongated form during active inward movement, and a more rounded shape during their less active, 'non-moving' phase. Similar regular changes of form have been observed previously by Gerisch (1964), studying aggregating cells of *Dictyostelium purpureum*. We detected no systematic difference in cell density between light areas of moving cells and darker areas of non-moving cells. The visible pattern presumably depends on differences in the light-scattering properties of the two types of area.

The width of bands of moving cells represents the distance the signal travels in the time that cells remain elongated after stimulation; it should therefore not vary with signal frequency. The interband distance, on the other hand, *should* depend on signal frequency since it reflects the distance that waves are propagated in the time elapsing between successive signals. This prediction can be tested since we observed that during the course of aggregation the period of signalling within a given territory decreases from 10 min to 3 min or less, by which time the cells are beginning to form streams and it becomes hard to discern individual movement steps (see also Durston, 1974). Table 1 shows results obtained by

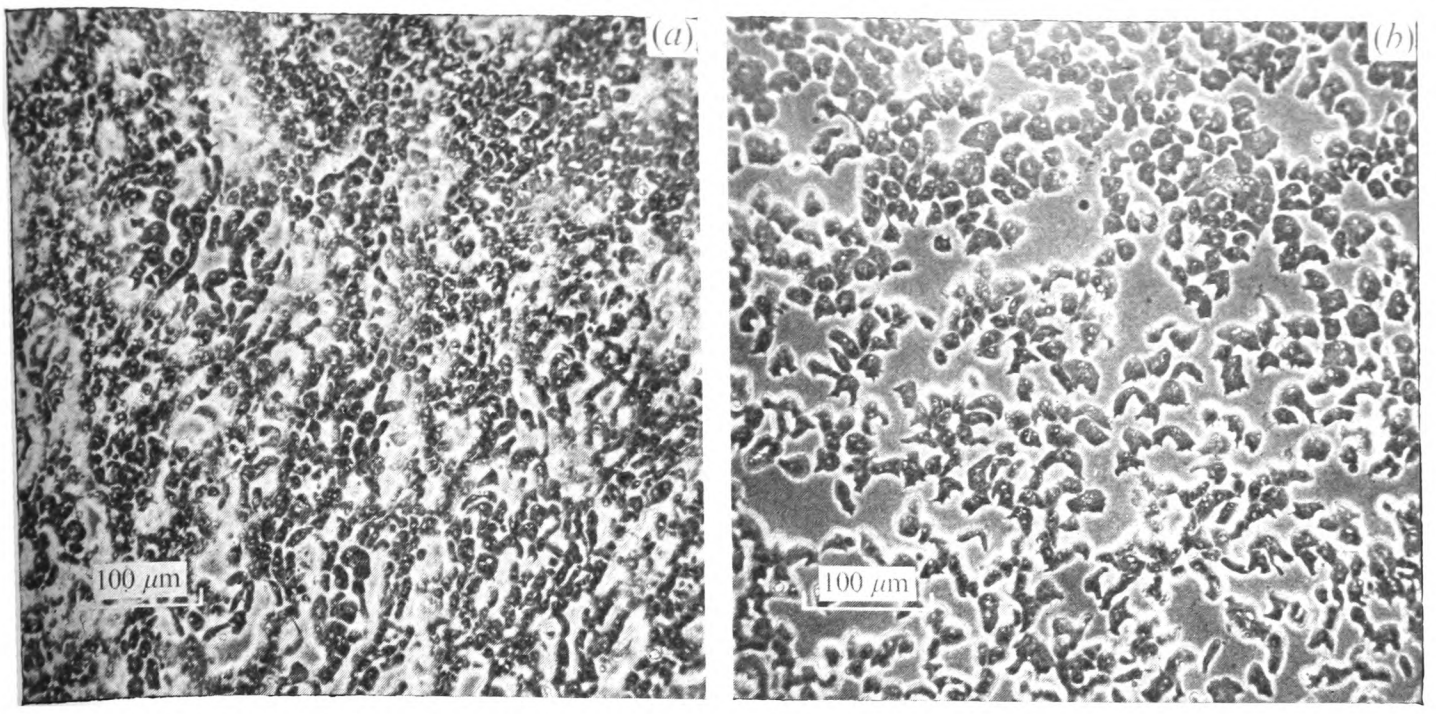

Fig. 2. The junction between a movement band and an interband in the concentric wave pattern of *D. discoideum* NC4. Cell densities: (*a*) 5×10^5 amoebae/cm^2; (*b*) $1{\cdot}25 \times 10^5$ amoebae/cm^2.

Table 1. *Variation of wave pattern with signal period in D. discoideum* NC4

Cell density* (amoebae/cm^2)	Signal period (s)	Movement band† (μm)	Interband (μm)	Wavelength‡ (μm)	Velocity§ (μm/s)
$1{\cdot}25 \times 10^5$ (32)	224	536	780	1316	5·9
	184	489	451	940	5·1
$1{\cdot}5 \times 10^5$ (29)	480	470	1645	2115	4·4
	330	470	1410	1880	5·7
	290	470	705	1175	4·1
	239	470	564	1034	4·3
$1{\cdot}7 \times 10^5$ (27)	300	705	752	1457	4·9
	290	658	658	1316	4·5
	285	658	611	1269	4·5
	275	658	658	1316	4·8
	270	658	611	1269	4·7
	260	611	752	1363	5·2
$2{\cdot}5 \times 10^5$ (22·5)	600	470	1645	2115	3·5
	530	470	1880	2350	4·4
	530	470	1457	1927	3·6
	330	470	940	1410	4·3
	310	470	658	1128	3·6
	285	470	752	1222	4·3
5×10^5 (16)	600	470	2021	2491	4·2
	580	470	2021	2491	4·3
	290	470	564	1034	3·6
	275	470	564	1034	3·8

* The intercellular distances, in μm, are given in parentheses.

† The velocity of signal propagation can be calculated by dividing the movement band widths in this column by 100 s, the movement duration (see text).

‡ Sum of the widths of a movement band and the corresponding interband.

§ Derived from wavelength/signal period.

Table 2. *Velocity of signal propagation determined by measuring the time interval between movement responses of individual cells*

Strain	Cell density* (amoebae/cm²)	Distance between chosen cells (μm)	Interval between responses† (s)	Velocity‡ (μm/s)
NC4	5×10^4 (51)	143	16(2)	8·9
		128	16(3)	8·0
		114	16(3)	7·1
		70	16	4·4
		50	12(7)	4·2
NC4	$1·25 \times 10^5$ (32)	230	32	7·2
		215	40	5·4
		138	24	5·8
		136	32	4·3
		130	28	4·6
		100	12	8·3
NC4	$1·5 \times 10^5$ (29)	180	32(4)	5·6
		115	24(3)	4·8
		115	20(3)	5·8
		108	24(3)	4·5
		108	20(2)	5·4
		108	16(2)	6·8
		186	34(2)	5·5
		100	17	5·9
		93	17(2)	5·5
		86	17	5·1
AX2	2×10^5 (25)	180	47(5)	3·8
		160	46(2)	3·5
NC4	4×10^5 (18)	255	64(8)	4·0
		210	68(8)	3·1
		158	32(8)	4·9
		115	32(8)	3·6
		65	20(8)	3·3
		74	12(8)	6·2
NC4	5×10^5 (16)	104	32	3·3
		104	28	3·7

* The intercellular distances, in μm, are given in parentheses.

† Where more than one determination has been made the number of determinations is indicated in parentheses.

‡ Since we show below that the signal is actually propagated in steps of about 57 μm this method of determining the velocity is subject to error, especially for pairs of cells that are close together.

microscopic observation (see Fig. 5) of fields exhibiting wave patterns. The signal period was measured by timing the interval between movements of a selected cell in response to two successive signal fronts. The width of the corresponding movement band was immediately recorded and the width of the corresponding interband measured by scanning the preparation outwards from the movement band. Variation in signal frequency affects the width of the interbands of non-moving cells, whereas the width of the movement bands is almost constant.

It is possible to derive several independent estimates of the velocity of signal propagation. First, since the wavelength corresponds to the distance over which a signal is propagated before the next signal leaves the centre, the velocity is given by dividing the wavelength by the signal period, as in Table 1. Second, if the width of the movement band corresponds to the distance the signal is propagated in the time cells remain elongated, the velocity of wave

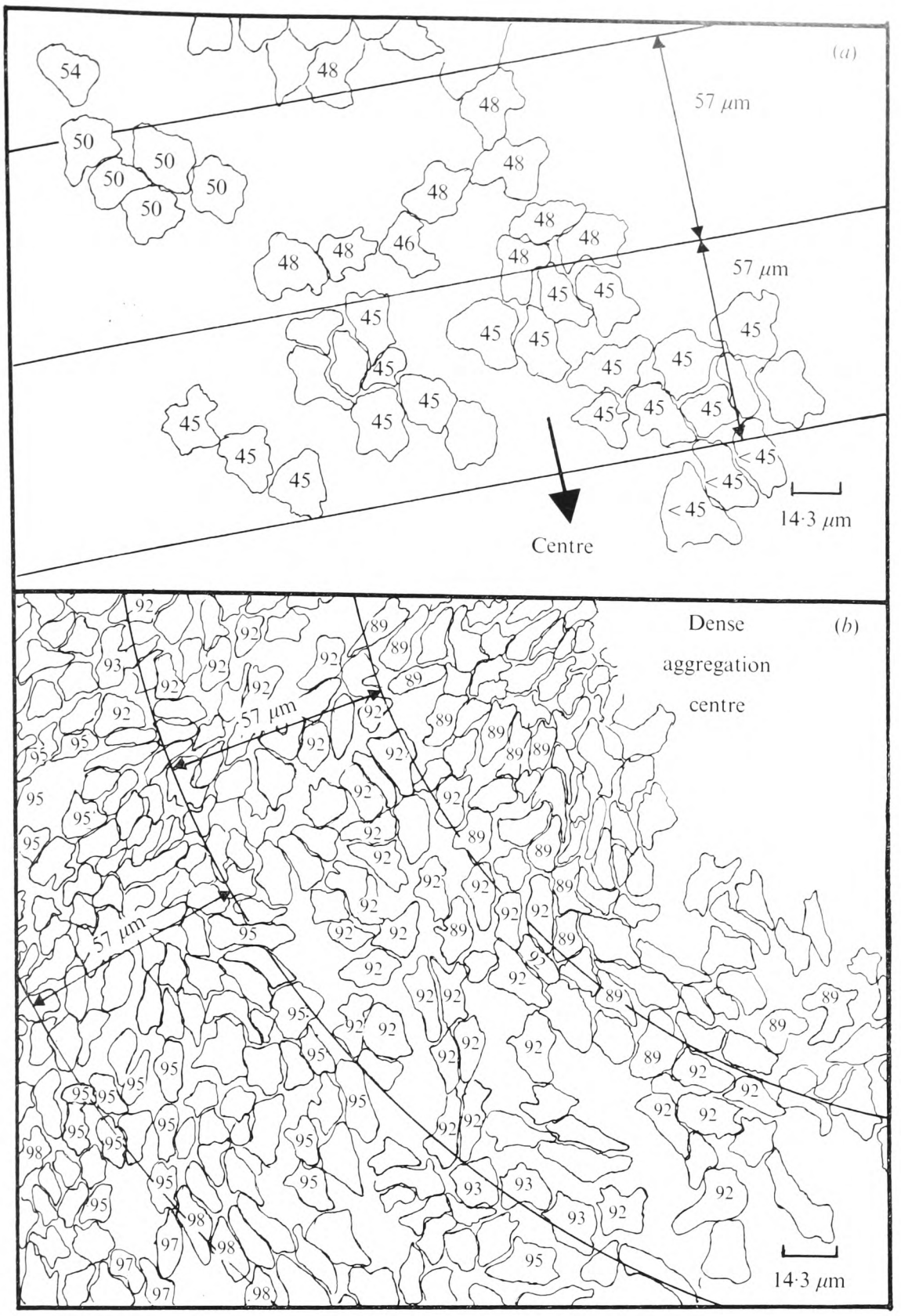

Fig. 3. The range of the relayed signal and the relay time. A field of amoebae was traced from a time-lapse film of aggregating *D. discoideum* NC4. The film was run repeatedly through the same sequence and the frame (frames at 4 s intervals) in which each amoeba started to move in response to a particular signal front was recorded; (*a*) and (*b*) correspond to independent experiments. In (*a*) unnumbered amoebae showed no marked movement response. In (*b*), not all of the amoebae were analysed and in many cases discrete movement steps could not be distinguished owing to the close proximity of the cells. Cell densities: (*a*) $2{\cdot}3 \times 10^5$ amoebae/cm^2; (*b*) $7{\cdot}3 \times 10^5$ amoebae/cm^2. The lines have been drawn knowing the position of the centre, and in such a way as to include as many 'simultaneously' responding amoebae as possible.

Table 3. *Variation of velocity of signal propagation with intercellular distance*

Cell density (amoebae/cm²)	Intercellular distance (μm)	Velocity* (μm/s)
5×10^4†	51	6·89 ± 0·67(3) 6·30 ± 0·42(7) 5·64 ± 0·41(5) 5·47 ± 0·39(16)
$1{\cdot}25 \times 10^5$	32	5·77 ± 0·18(43) 5·23 ± 0·20(7)
$1{\cdot}5 \times 10^5$	29	6·44 ± 0·40(7) 4·75 ± 0·10(10) 4·70‡(4)
$1{\cdot}9 \times 10^5$	26	5·28 ± 0·27(11)
$2{\cdot}5 \times 10^5$	22·5	6·48 ± 0·47(8) 5·63 ± 0·16(68) 4·76 ± 0·14(8)
5×10^5	16	5·51 ± 0·28(8) 4·70‡(5) 4·70‡(5)

* Measured movement band widths divided by 100 s, the movement duration (see above). The results are expressed as mean velocities with standard errors for 95 % confidence limits. Separate experiments are recorded on different lines. Number of determinations are in parentheses.

† The wave pattern in fields of this density is not visible to the naked eye but can be seen clearly under the microscope. Aggregation is delayed, compared with fields of higher densities, and can take up to 13 h.

‡ No variation was observed.

propagation would also be obtained by dividing this time into the movement-band width. We have measured the movement duration (maintenance of elongated shape) by analysis of time-lapse films and found a mean value of 99·5 (S.D. 14·1) s, in agreement with the value of 100 s reported by Cohen & Robertson (1971*b*). The velocities of signal propagation which would be obtained by dividing the movement-band widths in Table 1 by this value agree well with those listed in the last column. Finally, we determined the velocity by measuring the time interval between the movement responses of individual cells a known distance apart in the region of the advancing wave front (Table 2).

Range of relayed signal and relay time

If the signal emanating from a centre is propagated outwards by a succession of discrete relay steps (Shaffer, 1962; Gerisch, 1965; Cohen & Robertson, 1971*a*) its velocity should depend on the number of relay events per unit time and on the range of influence of each event. We have attempted to obtain independent measurements of these two parameters Figure 3 shows analyses of time-lapse films of aggregating *D. discoideum* NC4 where we have determined the film frame in which individual amoebae start to move in response to a signal propagating across the field. Two independent experiments were analysed. Blocks of amoebae measuring about 57 μm across generally respond in the same film frame (i.e. within 4 s of each other). This demonstrates signal relay directly, and shows that the range of the relayed signal is about 57 μm. The responses of successive blocks of amoebae are separated by two, or more frequently three, film frames. The relay time is therefore less than, but close to, 12 s. The range of influence of the relayed signal divided by the relay time yields a minimum estimate of the velocity of signal propagation (57 μm/12 s = 4·75 μm/s). This is in satisfactory agreement with the previous measurements of velocity.

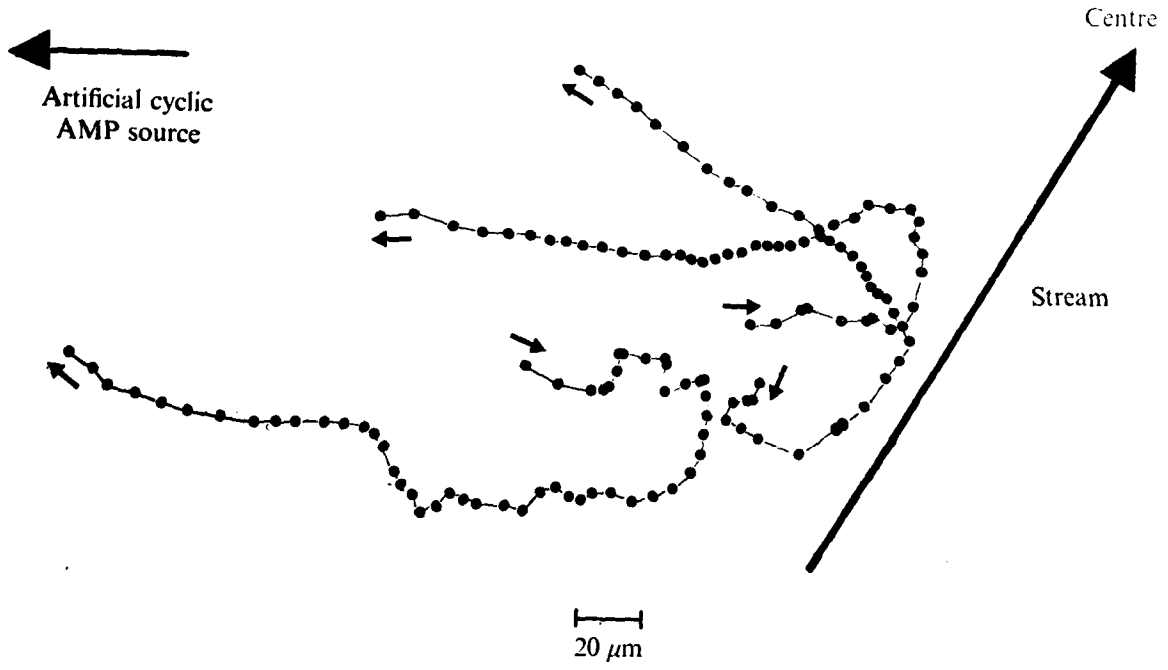

Fig. 4. Analysis of a time-lapse film showing the path tracks of three AX2 amoebae initially carrying out discrete movement steps towards a natural centre with a period of about 3 min. A small filter-paper square saturated with a cyclic AMP solution (2 μl of a 10^{-3} M solution) was placed in the field at a distance of 1 mm in the direction indicated. Within 25 min the amoebae were diverted from the natural centre by the artificial cyclic AMP source and moved continuously and with increasing speed towards it. Later, outside the field shown, their speed reached a maximum of 0·5 μm/s. The points represent the position of the centre of each cell at 60 s intervals.

Effect of cell density on velocity of signal propagation

Since the range of influence of the relayed signal as well as the relay time are approximately the same in the two populations analysed in Fig. 3, we have derived the same estimate of velocity for them. It is possible to evaluate more reliably the dependence of velocity of signal propagation on cell density from the data given in Tables 1 and 2 as these were obtained in populations with cell densities varying from 5×10^4 to 5×10^5 amoebae/cm^2. The corresponding intercellular distances (see Methods) range from 51 to 16 μm respectively. There is no marked dependence of velocity on density, but the velocity declines somewhat with increase in density. More extensive data point to the same conclusion (Table 3). Student's 't' test on the correlation coefficient (eqn 39, Bailey, 1972) between velocity and intercellular distance in each of the three tables indicated that the correlation for these two variables was significantly different from zero ($P \leqslant 0{\cdot}001$). We conclude that there is a dependence of velocity on density.

Refractory period for movement response

To ensure centripetal movement of responding cells and outward propagation of the signal, it would seem that cells must become refractory to stimulation for some time after being signalled (Gerisch, 1965). The refractory periods for the two components of the response, namely oriented movement and signal relay, need not be the same. Robertson *et al.* (1972) and Durston (1974) presented evidence for a refractory period for relay and showed that it decreases during aggregation from about 7 to 2 min.

Table 4. *Consecutive directional path times in cells stimulated from different directions*

Strain	Cell	Directional path time* (s)	Minimum directional path time (s)
NC4	1	24, 12, 20, 12, 16, 16, 20, 16	12
	2	16, 16, 12	12
	3	16, 12, 36, 16, 24, 32, 24, 20	12
AX2	4	12, 20, 32	12
	5	16, 20, 16, 12, 32, 44, 44, 28, 40, 28	12
	6	68, 16, 36, 28, 24, 60, 20, 32, 20, 28, 24, 20, 28, 32, 44, 40, 24	16
	7	12, 36, 36, 24, 16, 12, 56, 44, 56, 24, 32, 16, 120, 16, 20, 16	12
	8	16, 48, 48, 48	16

* The short stationary periods between directional displacements which occur in some directional changes were added to the path time in the new direction.

We have attempted to detect a refractory period for movement response. A cell responding periodically to a natural centre with a movement duration of 100 s can be made to move continuously towards a constant artificial cyclic AMP source (Fig. 4). The refractory period for movement response for the cells in Fig. 4, initially moving with a period of 3 min, was $\leqslant$ 100 s. We also attempted to measure the minimum unidirectional path time of cells whose movement response demonstrated that they were being signalled successively from different directions. By analysing successive frames of time-lapse films we have determined the directional path times of isolated cells in the neighbourhood of streams in an aggregation field. Such cells (cells 1 to 5, Table 4) first move obliquely to the stream, in the direction of the centre. After that they make further movement steps more nearly perpendicular to the stream and even biased away from the centre, as they are stimulated by the relayed signal passing down the stream. The directional path times for cells 6, 7 and 8 in Table 4 correspond to directional changes observed in the 'zig-zag' path tracks of cells which were approximately equidistant from two streams moving continuously towards an artificial cyclic AMP source.

The minimum unidirectional path time for individual cell tracks was most commonly 12 s (Table 4). We conclude therefore that the refractory period for movement response is probably not more than 12 s. However, we have also observed (unpublished) that 12 s is the minimum time required for cell displacement, that is, for contraction of the back of the cell following directional pseudopod formation. This approach therefore establishes only an upper limit for the refractory period for movement response.

DISCUSSION

Our interpretation of the concentric wave pattern analysed in this paper is given in Fig. 5. Near the bottom of the field is an aggregation centre, depicted formally as a single cell, to which other cells in the field are responding. The field is divided into concentric zones representing the discrete 57 μm steps by which signals are relayed out from the centre. The visible pattern depends on the alternation of bands of elongated moving cells (MB) and of relatively non-motile, rounded cells (IB). Each movement band comprises the cells responding at a given moment to a signal relayed out from the centre. The band itself moves outwards as

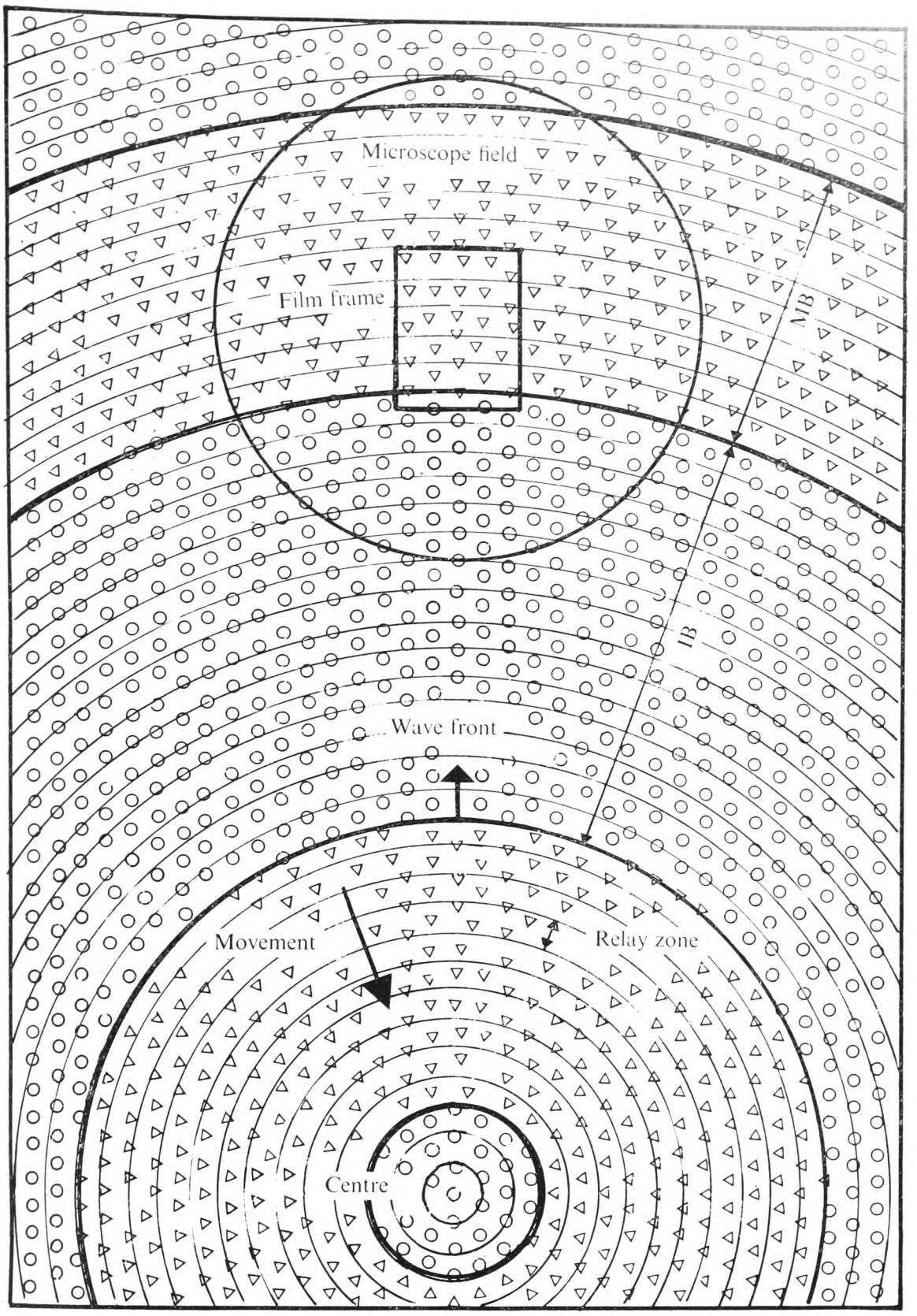

Fig. 5. Model of signal propagation. The field of amoebae is divided into concentric relay zones. Each relay zone is depicted as encompassing only one or two amoebae. This would be true for sparse aggregation territories. For denser territories see text and Fig. 3. Two wave fronts corresponding to two consecutive signals from the centre are shown propagating outwards. The direction of cell movement is inward as shown. The sizes of the microscope field and film frame used to collect the data presented in this work are indicated, in relation to the size of the aggregation territory as a whole. MB, movement band; IB, interband; △, moving cell; ○, non-moving cell.

cells in its most proximal zone complete their inward movement step and cells in its most distal zone relay the signal ahead of them. We have drawn each movement band as comprising 10 relay zones; this is the approximate number obtained by dividing the movement duration of 100 s by the relay time of slightly less than 12 s, as well as by dividing the movement band width (470 to 705 μm) by the range of influence of the relayed signal (57 μm). The value of 57 μm for signal range agrees well with our measurement of 51 μm for the maximum intercellular distance permitting aggregation to occur (unpublished observations; see also Konijn & Raper, 1961, Gerisch, 1961). With a signal propagation velocity of about 350 μm/min and an initial territory size of up to 10000 μm in radius, one signal could take 30 to 40 min to reach the edge of the aggregate; if the signalling period were 5 min, 6 to 8 signals would be propagating outwards at the same time (see Gerisch, 1968).

Cohen & Robertson (1971*a*) have calculated the delay between a cell's being signalled and its relaying the signal (relay delay time) to be 15 s. This is to be compared with our upper estimate of 12 s for the relay time, which includes this delay as well as the presumably very short time required for the signal to diffuse from one zone to the next. The calculation of Cohen & Robertson is based on the assumption that the signal is relayed from cell to cell (10 μm) in a dense population, and on the velocity of signal propagation of 43 μm/min obtained by Gerisch (1965). Our results show that in almost confluent populations the range of the relayed signal encompasses 4 to 6 cells, and Cohen & Robertson (1971*a*) have themselves reported 'near simultaneous pulsatile movement of entire areas of amoebae occurring during aggregation'. Gerisch (1965) was using multilayered populations, where signal range may well be decreased. Certainly our velocity of signal propagation of about 350 μm/min is considerably greater than the 43 μm/min observed by Gerisch (1965) for *D. discoideum* strain V12 and the 28 to 50 μm/min observed by Samuel (1961) for *D. purpureum*. On the other hand it is somewhat slower than the 500 μm/min observed in films by Bonner (cited in Shaffer, 1962). These differences may be due to the strain employed or to experimental conditions. The velocity of signal propagation decreases somewhat with increase in cell density (Tables 1, 2 and 3). We cannot account for this dependence but it is clear that the velocity of signal propagation could be affected by changes in the balance between cyclic AMP output (assuming that cyclic AMP is the signal) and phosphodiesterase activity (Cohen & Robertson, 1971*a*). It is also possible that a feedback control mechanism operates, e.g. cyclic AMP output per cell may be inversely related to cell density due to control exerted by external cyclic AMP (Shaffer, 1962).

The minimum refractory period required to ensure centripetal cell movement in uniform fields of amoebae can be derived by the following argument, using our upper limit of 12 s for the relay time. A cell stimulated at time $t = 0$ s must become refractory at some time before emitting its own pulse of cyclic AMP i.e. by $t = 12$ s; it must remain refractory until cells in the relay zone distal to it have themselves signalled (at $t = 24$ s), and until that signal has been degraded by phosphodiesterase to below threshold level. Since Cohen & Robertson (1971*b*) have calculated the duration of the chemotactic signal to be a fraction of a second, the minimum refractory period would be about 12 s. We have analysed the movement behaviour of single cells in the neighbourhood of streams. They showed several changes of direction, presumably due to successive stimulations by a signal propagating down the stream. From the minimum directional path time of these cells we have concluded that the upper limit to the cell refractory period for movement response is 12 s. It is therefore close to the minimum tolerable duration of the refractory period. Given a short refractory period for movement response, the net effect of individual cells sensing a signal passing down a stream would be to draw them into the stream. This would explain the

previously somewhat puzzling observations of Shaffer (1957*a*), that responding cells appear to approach a stream perpendicularly rather than biased towards the centre.

There remains the question of why cells in early aggregation remain elongated for 100 s when the signal is of very short duration. We have observed that the movement step of cells travelling in one direction frequently shows a fast component lasting for around 20 to 40 s, followed by progressively slower movement until the cell comes to rest (unpublished observations). The latter phase could represent residual directional movement and elongation that is maintained unless the cell is otherwise stimulated.

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